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(54) Title: M. TUBERCULOSIS ANTIGENS

(57) Abstract: The present invention is based on the identification and characterization of a number of novel *M. tuberculosis* derived proteins and protein fragments. The invention is directed to the polypeptides and immunologically active fragments thereof, the genes encoding them, immunological compositions such as vaccines and skin test reagents containing the polypeptides.

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M. TUBERCULOSIS ANTIGENS

Field of invention

The present invention discloses new immunogenic polypeptides and new immunogenic
5 compositions based on polypeptides derived from the short time culture filtrate of *M. tuberculosis*.

General Background

Human tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is a severe
global health problem, responsible for approx. 3 million deaths annually, according to the
10 WHO. The world-wide incidence of new tuberculosis (TB) cases had been falling during
the 1960s and 1970s but during recent years this trend has markedly changed in part due
to the advent of AIDS and the appearance of multidrug resistant strains of *M. tuberculosis*.

The only vaccine presently available for clinical use is BCG, a vaccine whose efficacy re-
15 mains a matter of controversy. BCG generally induces a high level of acquired resistance
in animal models of TB, but several human trials in developing countries have failed to
demonstrate significant protection. Notably, BCG is not approved by the FDA for use in
the United States because BCG vaccination impairs the specificity of the Tuberculin skin
test for diagnosis of TB infection.

20

This makes the development of a new and improved vaccine against TB an urgent matter,
which has been given a very high priority by the WHO. Many attempts to define protective
mycobacterial substances have been made, and different investigators have reported in-
creased resistance after experimental vaccination. However, the demonstration of a spe-
25 cific long-term protective immune response with the potency of BCG has not yet been
achieved.

Immunity to *M. tuberculosis* is characterized by some basic features; specifically sensi-
tized T lymphocytes mediates protection, and the most important mediator molecule
30 seems to be interferon gamma (IFN- γ).

M. tuberculosis holds, as well as secretes, several proteins of potential relevance for the
generation of a new TB vaccine. For a number of years, a major effort has been put into

the identification of new protective antigens for the development of a novel vaccine against TB. The search for candidate molecules has primarily focused on proteins released from dividing bacteria. Despite the characterization of a large number of such proteins only a few of these have been demonstrated to induce a protective immune response as subunit vaccines in animal models, most notably ESAT-6 and Ag85B (Brandt et al 2000).

In 1998 Cole et al published the complete genome sequence of *M. tuberculosis* and predicted the presence of approximately 4000 open reading frames (Cole et al 1998). Among others, nucleotide sequences comprising Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878 and Rv3879c are described, and putative protein sequences for the above sequences are suggested. However important, this sequence information cannot be used to predict if the DNA is translated and expressed as proteins *in vivo*. More importantly, it is not possible on the basis of the sequences to predict whether a given sequence will encode an immunogenic or an inactive protein. The only way to determine if a protein is recognized by the immune system during or after an infection with *M. tuberculosis* is to produce the given protein and test it in an appropriate assay as described herein.

Diagnosing *M. tuberculosis* infection in its earliest stage is important for effective treatment of the disease. Current diagnostic assays to determine *M. tuberculosis* infection are expensive and labour-intensive. In the industrialized part of the world the majority of patients exposed to *M. tuberculosis* receive chest x-rays and attempts are made to culture the bacterium *in vitro* from sputum samples. X-rays are insensitive as a diagnostic assay and can only identify infections in a very progressed stage. Culturing of *M. tuberculosis* is also not ideal as a diagnostic tool, since the bacteria grows poorly and slowly outside the body, which can produce false negative test results and take weeks before results are obtained. The standard tuberculin skin test is an inexpensive assay, used in third world countries, however it is far from ideal in detecting infection because it cannot distinguish *M. tuberculosis*-infected individuals from *M. bovis* BCG-vaccinated individuals and therefore cannot be used in areas of the world where patients receive or have received childhood vaccination with bacterial strains related to *M. tuberculosis*, e.g. a BCG vaccination.

Animal tuberculosis is caused by *Mycobacterium bovis*, which is closely related to *M. tuberculosis* and within the tuberculosis complex. *M. bovis* is an important pathogen that can infect a range of hosts, including cattle and humans. Tuberculosis in cattle is a major

cause of economic loss and represents a significant cause of zoonotic infection. A number of strategies have been employed against bovine TB, but the approach has generally been based on government-organized programs by which animals deemed positive to defined screening test are slaughtered. The most common test used in cattle is Delayed-type hypersensitivity with PPD as antigen, but alternative *in vitro* assays are also developed. However, investigations have shown the both the *in vivo* and the *in vitro* tests have a relative low specificity, and the detection of false-positive is a significant economic problem (Pollock et al 2000). There is therefore a great need for a more specific diagnostic reagent, which can be used either *in vivo* or *in vitro* to detect *M. bovis* infections in animals.

Summary of the invention

The invention is related to preventing, treating and detecting infections caused by species of the tuberculosis complex (*M. tuberculosis*, *M. bovis*, *M. africanum*) by the use of a polypeptide comprising a *M. tuberculosis* antigen or an immunogenic portion or other variant thereof, or by the use of a DNA sequence encoding a *M. tuberculosis* antigen or an immunogenic portion or other variant thereof.

Detailed disclosure of the invention

The present invention discloses a substantially pure polypeptide, which comprises an amino acid sequence selected from

- (a) Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1;
- (b) an immunogenic portion, e.g. a T-cell epitope, of any one of the sequences in (a); and /or
- (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic.

Preferably, the amino acid sequence analogue has at least 80%, more preferred at least 90% and most preferred at least 95% sequence identity to any one of the sequences in (a) or (b).

The invention further discloses a fusion polypeptide, which comprises an amino acid sequence selected from

- (a) Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1
- (b) an immunogenic portion, e.g. a T-cell epitope, of any one of the sequences in (a); and /or
- 5 (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic; and at least one fusion partner.

Preferably, the fusion partner comprises a polypeptide fragment selected from

- 10 (a) a polypeptide fragment derived from a virulent mycobacterium, such as ESAT-6, MPB64, MPT64, TB10.4, CFP10, RD1-ORF5, RD1-ORF2, Rv1036, Ag85A, Ag85B, Ag85C, 19kDa lipoprotein, MPT32, MPB59 and alpha-crystallin;
- (b) a polypeptide according to the invention and defined above and/or
- (c) at least one immunogenic portion, e.g. a T-cell epitope, of any of such polypep-
- 15 tides in (a) or (b)

The invention further relates to a polypeptide, which comprises an amino acid sequence selected from

- (a) Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1
- 20 (b) an immunogenic portion, e.g. a T-cell epitope, of any one of the sequences in (a); and /or
- (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;
- 25 which is lipidated so as to allow a self-adjuvating effect of the polypeptide.

Further, the invention relates to a polypeptide, which comprises an amino acid sequence selected from

- (a) Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1
- 30 (b) an immunogenic portion, e.g. a T-cell epitope, of any one of the sequences in (a); and /or
- (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;
- 35 for use as a vaccine, as a pharmaceutical or as a diagnostic reagent.

In another embodiment, the invention relates to the use of a polypeptide as defined above for the preparation of a pharmaceutical composition for diagnosis, e.g. for diagnosis of tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, and the use of a polypeptide as defined above for the preparation of a pharmaceutical composition, e.g. for the vaccination against infection caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

- 10 In a still further embodiment, the invention relates to an immunogenic composition comprising a polypeptide as defined above, preferably in the form of a vaccine or in the form of a skin test reagent.

In another embodiment, the invention relates to a nucleic acid fragment in isolated form
15 which

- (a) comprises a nucleic acid sequence which encodes a polypeptide as defined above, or comprises a nucleic acid sequence complementary thereto; or
- (b) has a length of at least 10 nucleotides and hybridizes readily under stringent hybridization conditions with a nucleotide sequence selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1 nucleotide sequences or a sequence complementary thereto, or with a nucleotide
20 sequence selected from a sequence in (a)

The nucleic acid fragment is preferably a DNA fragment. The fragment can be used as a
25 pharmaceutical.

In one embodiment, the invention relates to a vaccine comprising a nucleic acid fragment according to the invention, optionally inserted in a vector, the vaccine effecting *in vivo* expression of antigen by an animal, including a human being, to whom the vaccine has been
30 administered, the amount of expressed antigen being effective to confer substantially increased resistance to tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, in an animal, including a human being.

In a further embodiment, the invention relates to the use of a nucleic acid fragment according to the invention for the preparation of a composition for the diagnosis of tuberculosis caused by virulent mycobacteria, e. g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, and the use of a nucleic acid fragment according
5 to the invention for the preparation of a pharmaceutical composition for the vaccination against tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

In a still further embodiment, the invention relates to a vaccine for immunizing an animal,
10 including a human being, against tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, comprising as the effective component a non-pathogenic microorganism, wherein at least one copy of a DNA fragment comprising a DNA sequence encoding a polypeptide as defined above has been incorporated into the microorganism (e.g. placed on a plasmid or in the
15 genome) in a manner allowing the microorganism to express and optionally secrete the polypeptide.

In another embodiment, the invention relates to a replicable expression vector, which comprises a nucleic acid fragment according to the invention, and a transformed cell har-
20 bouring at least one such vector.

In another embodiment, the invention relates to a method for producing a polypeptide as defined above, comprising

- (a) inserting a nucleic acid fragment according to the invention into a vector which is
25 able to replicate in a host cell, introducing the resulting recombinant vector into the host cell, culturing the host cell in a culture medium under conditions sufficient to effect expression of the polypeptide, and recovering the polypeptide from the host cell or culture medium;
- (b) isolating the polypeptide from a whole mycobacterium, e.g. *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, from culture filtrate or
30 from lysates or fractions thereof; or
- (c) synthesizing the polypeptide e.g. by solid or liquid phase peptide synthesis.

The invention also relates to a method of diagnosing tuberculosis caused by virulent my-
35 cobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Myco-*

bacterium bovis, in an animal, including a human being, comprising intradermally injecting, in the animal, a polypeptide as defined above or an immunogenic composition as defined above, a positive skin response at the location of injection being indicative of the animal having tuberculosis, and a negative skin response at the location of injection being
5 indicative of the animal not having tuberculosis.

In another embodiment, the invention relates to a method for immunizing an animal, including a human being, against tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, comprising
10 administering to the animal the polypeptide as defined above, the immunogenic composition according to the invention, or the vaccine according to the invention.

Another embodiment of the invention relates to a monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide as defined above in an immuno assay, or
15 a specific binding fragment of said antibody. Preferably, said antibody is for use as a diagnostic reagent, e.g. for detection of mycobacterial antigens in sputum, urine or other body fluids of an infected animal, including a human being.

In a further embodiment the invention relates to a pharmaceutical composition which
20 comprises an immunologically responsive amount of at least one member selected from the group consisting of:

- (a) a polypeptide selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1, or an immunogenic portion thereof;
- 25 (b) an amino acid sequence which has a sequence identity of at least 70% to any one of said polypeptides in (a) and is immunogenic;
- (c) a fusion polypeptide comprising at least one polypeptide or amino acid sequence according to (a) or (b) and at least one fusion partner;
- (d) a nucleic acid sequence which encodes a polypeptide or amino acid sequence
30 according to (a), (b) or (c);
- (e) a nucleic acid sequence which is complementary to a sequence according to (d);
- (f) a nucleic acid sequence which has a length of at least 10 nucleotides and which hybridizes under stringent conditions with a nucleic acid sequence according to (d) or (e); and

- (g) a non-pathogenic micro-organism which has incorporated (e.g. placed on a plasmid or in the genome) therein a nucleic acid sequence according to (d), (e) or (f) in a manner to permit expression of a polypeptide encoded thereby.
- 5 In a still further embodiment the invention relates to a method for stimulating an immunogenic response in an animal which comprises administering to said animal an immunologically stimulating amount of at least one member selected from the group consisting of:
- (a) a polypeptide selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195,
10 Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1, or an immunogenic portion thereof;
- (b) an amino acid sequence which has a sequence identity of at least 70% to any one of said polypeptides in (a) and is immunogenic;
- (c) a fusion polypeptide comprising at least one polypeptide or amino acid sequence
15 according to (a) or (b) and at least one fusion partner;
- (d) a nucleic acid sequence which encodes a polypeptide or amino acid sequence according to (a), (b) or (c);
- (e) a nucleic acid sequence which is complementary to a sequence according to (d);
- (f) a nucleic acid sequence which has a length of at least 10 nucleotides and which
20 hybridizes under stringent conditions with a nucleic acid sequence according to (d) or (e); and
- (g) a non-pathogenic micro-organism which has incorporated therein (e.g. placed on a plasmid or in the genome) a nucleic acid sequence according to (d), (e) or (f) in a manner to permit expression of a polypeptide encoded thereby.
- 25 The vaccine, immunogenic composition and pharmaceutical composition according to the invention can be used prophylactically in a subject not infected with a virulent mycobacterium; or therapeutically in a subject already infected with a virulent mycobacterium.
- 30 The invention also relates to a method for diagnosing previous or ongoing infection with a virulent mycobacterium, said method comprising
- (a) contacting a sample, e.g. a blood sample, with a composition comprising an antibody according to the invention, a nucleic acid fragment according to the invention and/or a polypeptide as defined above, or

- (b) contacting a sample, e.g. a blood sample comprising mononuclear cells (e.g. T-lymphocytes), with a composition comprising one or more polypeptides as defined above in order to detect a positive reaction, e.g. proliferation of the cells or release of cytokines such as IFN- γ .

5

Finally, the invention relates to a method of diagnosing *Mycobacterium tuberculosis* infection in a subject comprising:

- (a) contacting a polypeptide as defined above with a bodily fluid of the subject;
(b) detecting binding of an antibody to said polypeptide, said binding being an indication that said subject is infected by *Mycobacterium tuberculosis* or is susceptible to *Mycobacterium tuberculosis* infection.

10

Definitions

The word "polypeptide" in the present invention should have its usual meaning. That is an amino acid chain of any length, including a full-length protein, oligopeptides, short peptides and fragments thereof, wherein the amino acid residues are linked by covalent peptide bonds.

The polypeptide may be chemically modified by being glycosylated, by being lipidated (e.g. by chemical lipidation with palmitoyloxy succinimide as described by Mowat et al. 1991 or with dodecanoyl chloride as described by Lustig et al. 1976), by comprising prosthetic groups, or by containing additional amino acids such as e.g. a his-tag or a signal peptide.

Each polypeptide may thus be characterised by specific amino acids and be encoded by specific nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant or synthetic methods wherein such polypeptide sequences have been modified by substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide and still be immunogenic in any of the biological assays described herein. Substitutions are preferably "conservative". These are defined according to the following table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other. The amino acids in the third column are indicated in one-letter code.

25

30

ALIPHATIC	Non-polar	GAP
		ILV
	Polar-uncharged	CSTM
		NQ
	Polar-charged	DE
		KR
AROMATIC		HFWY

A preferred polypeptide within the present invention is an immunogenic antigen from *M. tuberculosis*. Such antigen can for example be derived from *M. tuberculosis* and/or *M. tuberculosis* culture filtrate. Thus, a polypeptide comprising an immunogenic portion of one
5 of the above antigens may consist entirely of the immunogenic portion, or may contain additional sequences. The additional sequences may be derived from the native *M. tuberculosis* antigen or be heterologous and such sequences may, but need not, be immunogenic.

10 Each polypeptide is encoded by a specific nucleic acid sequence. It will be understood that such sequences include analogues and variants hereof wherein such nucleic acid sequences have been modified by substitution, insertion, addition or deletion of one or more nucleic acid. Substitutions are preferably silent substitutions in the codon usage which will not lead to any change in the amino acid sequence, but may be introduced to
15 enhance the expression of the protein.

In the present context the term "substantially pure polypeptide fragment" means a polypeptide preparation which contains at most 5% by weight of other polypeptide material with which it is natively associated (lower percentages of other polypeptide material are
20 preferred, e.g. at most 4%, at most 3%, at most 2%, at most 1%, and at most ½%). It is preferred that the substantially pure polypeptide is at least 96% pure, *i.e.* that the polypeptide constitutes at least 96% by weight of total polypeptide material present in the preparation, and higher percentages are preferred, such as at least 97%, at least 98%, at least 99%, at least 99,25%, at least 99,5%, and at least 99,75%. It is especially preferred
25 that the polypeptide fragment is in "essentially pure form", *i.e.* that the polypeptide fragment is essentially free of any other antigen with which it is natively associated, *i.e.* free of

any other antigen from bacteria belonging to the tuberculosis complex or a virulent mycobacterium. This can be accomplished by preparing the polypeptide fragment by means of recombinant methods in a non-mycobacterial host cell as will be described in detail below, or by synthesizing the polypeptide fragment by the well-known methods of solid or liquid
5 phase peptide synthesis, e.g. by the method described by Merrifield or variations thereof.

By the term "virulent mycobacterium" is understood a bacterium capable of causing the tuberculosis disease in an animal or in a human being. Examples of virulent mycobacteria are *M. tuberculosis*, *M. africanum*, and *M. bovis*. Examples of relevant animals are cattle, possums, badgers and kangaroos.

10

By "a TB patient" is understood an individual with culture or microscopically proven infection with virulent mycobacteria, and/or an individual clinically diagnosed with TB and who is responsive to anti-TB chemotherapy. Culture, microscopy and clinical diagnosis of TB are well known by any person skilled in the art.

15

By the term "PPD-positive individual" is understood an individual with a positive Mantoux test or an individual where PPD induces a positive *in vitro* recall response determined by release of IFN- γ .

20 By the term "delayed type hypersensitivity reaction" (DTH) is understood a T-cell mediated inflammatory response elicited after the injection of a polypeptide into, or application to, the skin, said inflammatory response appearing 72-96 hours after the polypeptide injection or application.

25 By the term "IFN- γ " is understood interferon-gamma. The measurement of IFN- γ is used as an indication of an immunological response.

By the terms "nucleic acid fragment" and "nucleic acid sequence" are understood any nucleic acid molecule including DNA, RNA, LNA (locked nucleic acids), PNA, RNA, dsRNA
30 and RNA-DNA-hybrids. Also included are nucleic acid molecules comprising non-naturally occurring nucleosides. The term includes nucleic acid molecules of any length, e.g. from 10 to 10000 nucleotides, depending on the use. When the nucleic acid molecule is for use as a pharmaceutical, e.g. in DNA therapy, or for use in a method for producing a polypeptide according to the invention, a molecule encoding at least one epitope is preferably
35 used, having a length from about 18 to about 1000 nucleotides, the molecule being op-

tionally inserted into a vector. When the nucleic acid molecule is used as a probe, as a primer or in antisense therapy, a molecule having a length of 10-100 is preferably used. According to the invention, other molecule lengths can be used, for instance a molecule having at least 12, 15, 21, 24, 27, 30, 33, 36, 39, 42, 50, 60, 70, 80, 90, 100, 200, 300, 5 400, 500 or 1000 nucleotides (or nucleotide derivatives), or a molecule having at most 10000, 5000, 4000, 3000, 2000, 1000, 700, 500, 400, 300, 200, 100, 50, 40, 30 or 20 nucleotides (or nucleotide derivatives). It should be understood that these numbers can be freely combined to produce ranges.

- 10 The term "stringent" when used in conjunction with hybridization conditions is as defined in the art, i.e. the hybridization is performed at a temperature not more than 15-20°C under the melting point T_m , cf. Sambrook et al, 1989, pages 11.45-11.49. Preferably, the conditions are "highly stringent", i.e. 5-10°C under the melting point T_m .
- 15 Throughout this specification, unless the context requires otherwise, the word "comprise", or variations thereof such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.
- 20 The term "sequence identity" indicates a quantitative measure of the degree of homology between two amino acid sequences of equal length or between two nucleotide sequences of equal length. If the two sequences to be compared are not of equal length, they must be aligned to best possible fit possible with the insertion of gaps or alternatively truncation at the ends of the protein sequences. The sequence identity can be calculated as
- 25 $\frac{(N_{ref} - N_{dif})100}{N_{ref}}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA se-
- 30 quence AGTCAGTC ($N_{dif}=2$ and $N_{ref}=8$). Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W. R. and D. J. Lipman (1988))(www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one aspect of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at <http://www2.ebi.ac.uk/clustalw/>.

A preferred minimum percentage of sequence identity is at least 80%, such as at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and at least 99.5%.

5 In a preferred embodiment of the invention, the polypeptide comprises an immunogenic portion of the polypeptide, such as an epitope for a B-cell or T-cell. The immunogenic portion of a polypeptide is a part of the polypeptide, which elicits an immune response in an animal or a human being, and/or in a biological sample determined by any of the biological assays described herein. The immunogenic portion of a polypeptide may be a T-cell
10 epitope or a B-cell epitope. Immunogenic portions can be related to one or a few relatively small parts of the polypeptide, they can be scattered throughout the polypeptide sequence or be situated in specific parts of the polypeptide. For a few polypeptides epitopes have even been demonstrated to be scattered throughout the polypeptide covering the full sequence (Ravn et al 1999).

15

In order to identify relevant T-cell epitopes which are recognised during an immune response, it is possible to use a "brute force" method: Since T-cell epitopes are linear, deletion mutants of the polypeptide will, if constructed systematically, reveal what regions of the polypeptide are essential in immune recognition, e.g. by subjecting these deletion
20 mutants e.g. to the IFN- γ assay described herein. Another method utilises overlapping oligopeptides for the detection of MHC class II epitopes, preferably synthetic, having a length of e.g. 20 amino acid residues derived from the polypeptide. These peptides can be tested in biological assays (e.g. the IFN- γ assay as described herein) and some of these will give a positive response (and thereby be immunogenic) as evidence for the
25 presence of a T cell epitope in the peptide. For the detection of MHC class I epitopes it is possible to predict peptides that will bind (Stryhn et al. 1996) and hereafter produce these peptides synthetic and test them in relevant biological assays e.g. the IFN- γ assay as described herein. The peptides preferably having a length of e.g. 8 to 11 amino acid residues derived from the polypeptide. B-cell epitopes can be determined by analysing the B
30 cell recognition to overlapping peptides covering the polypeptide of interest as e.g. described in Harboe et al 1998.

Although the minimum length of a T-cell epitope has been shown to be at least 6 amino acids, it is normal that such epitopes are constituted of longer stretches of amino acids.

35 Hence, it is preferred that the polypeptide fragment of the invention has a length of at

least 7 amino acid residues, such as at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, and at least 30 amino acid residues. Hence, in important embodiments of the inventive method, it is preferred that the polypeptide fragment has a length of at most 50 amino acid residues, such as at most 40, 35, 30, 25, and 20 amino acid residues. It should be understood that these numbers can be freely combined to produce ranges.

It is expected that the peptides having a length of between 10 and 20 amino acid residues will prove to be most efficient as MHC class II epitopes and therefore especially preferred lengths of the polypeptide fragment used in the inventive method are 18, such as 15, 14, 13, 12 and even 11 amino acid residues. It is expected that the peptides having a length of between 7 and 12 amino acid residues will prove to be most efficient as MHC class I epitopes and therefore especially preferred lengths of the polypeptide fragment used in the inventive method are 11, such as 10, 9, 8 and even 7 amino acid residues.

Immunogenic portions of polypeptides may be recognised by a broad part (high frequency) or by a minor part (low frequency) of the genetically heterogenic human population. In addition some immunogenic portions induce high immunological responses (dominant), whereas others induce lower, but still significant, responses (subdominant). High frequency > low frequency can be related to the immunogenic portion binding to widely distributed MHC molecules (HLA type) or even by multiple MHC molecules (Kilgus et al. 1991, Sinigaglia et al 1988).

In the context of providing candidate molecules for a new vaccine against tuberculosis, the subdominant epitopes are however as relevant as are the dominant epitopes since it has been shown (Olsen et al 2000) that such epitopes can induce protection regardless of being subdominant.

A common feature of the polypeptides of the invention is their capability to induce an immunological response as illustrated in the examples. It is understood that a variant of a polypeptide of the invention produced by substitution, insertion, addition or deletion is also immunogenic determined by any of the assays described herein.

An immune individual is defined as a person or an animal, which has cleared or controlled an infection with virulent mycobacteria or has received a vaccination with *M. bovis* BCG.

An immunogenic polypeptide is defined as a polypeptide that induces an immune response in a biological sample or an individual currently or previously infected with a virulent mycobacterium. The immune response may be monitored by one of the following 5 methods:

- An *in vitro* cellular response is determined by release of a relevant cytokine such as IFN- γ , from lymphocytes withdrawn from an animal or human being currently or previously infected with virulent mycobacteria, or by detection of proliferation of these T cells. The induction being performed by the addition of the polypeptide or the immunogenic portion to a suspension comprising from 1×10^5 cells to 3×10^5 cells per well. The cells being isolated from either the blood, the spleen, the liver or the lung and the addition of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μg per ml suspension and the stimulation being performed from two to five days. For monitoring cell proliferation the cells are pulsed with radioactive labeled Thymidine and after 16-22 hours of incubation detecting the proliferation by liquid scintillation counting. A positive response being a response more than background plus two standard derivations. The release of IFN- γ can be determined by the ELISA method, which is well known to a person skilled in the art. A positive response being a response more than background plus two standard derivations. Other cytokines than IFN- γ could be relevant when monitoring the immunological response to the polypeptide, such as IL-12, TNF- α , IL-4, IL-5, IL-10, IL-6, TGF- β . Another and more sensitive method for determining the presence of a cytokine (e.g. IFN- γ) is the ELISPOT method where the cells isolated from either the blood, the spleen, the liver or the lung are diluted to a concentration of preferable of 1 to 4×10^5 cells /ml and incubated for 18-22 hrs in the presence of of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μg per ml. The cell suspensions are hereafter diluted to 1 to 2×10^6 / ml and transferred to Maxisorp plates coated with anti-IFN- γ and incubated for preferably 4 to 16 hours. The IFN- γ producing cells are determined by the use of labeled secondary anti-IFN- γ antibody and a relevant substrate giving rise to spots, which can be enumerated using a dissection microscope. It is also a possibility to determine the presence of mRNA coding for the relevant cytokine by the use of the PCR technique. Usually one or more cytokines will be measured

utilizing for example the PCR, ELISPOT or ELISA. It will be appreciated by a person skilled in the art that a significant increase or decrease in the amount of any of these cytokines induced by a specific polypeptide can be used in evaluation of the immunological activity of the polypeptide.

5

- An *in vitro* cellular response may also be determined by the use of T cell lines derived from an immune individual or an *M. tuberculosis* infected person where the T cell lines have been driven with either live mycobacteria, extracts from the bacterial cell or culture filtrate for 10 to 20 days with the addition of IL-2. The induction being performed by addition of not more than 20 µg polypeptide per ml suspension to the T cell lines containing from 1×10^5 cells to 3×10^5 cells per well and incubation being performed from two to six days. The induction of IFN-γ or release of another relevant cytokine is detected by ELISA. The stimulation of T cells can also be monitored by detecting cell proliferation using radioactively labeled Thymidine as described above. For both assays a positive response being a response more than background plus two standard derivations.
- An *in vivo* cellular response which may be determined as a positive DTH response after intradermal injection or local application patch of at most 100 µg of the polypeptide or the immunogenic portion to an individual who is clinically or subclinically infected with a virulent Mycobacterium, a positive response having a diameter of at least 5 mm 72-96 hours after the injection or application.
- An *in vitro* humoral response is determined by a specific antibody response in an immune or infected individual. The presence of antibodies may be determined by an ELISA technique or a Western blot where the polypeptide or the immunogenic portion is absorbed to either a nitrocellulose membrane or a polystyrene surface. The serum is preferably diluted in PBS from 1:10 to 1:100 and added to the absorbed polypeptide and the incubation being performed from 1 to 12 hours. By the use of labeled secondary antibodies the presence of specific antibodies can be determined by measuring the OD e.g. by ELISA where a positive response is a response of more than background plus two standard derivations or alternatively a visual response in a Western blot.

- Another relevant parameter is measurement of the protection in animal models induced after vaccination with the polypeptide in an adjuvant or after DNA vaccination. Suitable animal models include primates, guinea pigs or mice, which are challenged with an infection of a virulent *Mycobacterium*. Readout for induced protection could be decrease of the bacterial load in target organs compared to non-vaccinated animals, prolonged survival times compared to non-vaccinated animals and diminished weight loss compared to non-vaccinated animals.

In general, *M. tuberculosis* antigens, and DNA sequences encoding such antigens, may be prepared using any one of a variety of procedures. They may be purified as native proteins from the *M. tuberculosis* cell or culture filtrate by procedures such as those described above. Immunogenic antigens may also be produced recombinantly using a DNA sequence encoding the antigen, which has been inserted into an expression vector and expressed in an appropriate host. Examples of host cells are *E. coli*. The polypeptides or immunogenic portion hereof can also be produced synthetically having fewer than about 100 amino acids, and generally fewer than 50 amino acids and may be generated using techniques well known to those ordinarily skilled in the art, such as commercially available solid-phase techniques where amino acids are sequentially added to a growing amino acid chain.

20

In the construction and preparation of plasmid DNA encoding the polypeptide as defined for DNA vaccination a host strain such as *E. coli* can be used. Plasmid DNA can then be prepared from overnight cultures of the host strain carrying the plasmid of interest, and purified using e.g. the Qiagen Giga -Plasmid column kit (Qiagen, Santa Clarita, CA, USA) including an endotoxin removal step. It is essential that plasmid DNA used for DNA vaccination is endotoxin free.

The immunogenic polypeptides may also be produced as fusion proteins, by which methods superior characteristics of the polypeptide of the invention can be achieved. For instance, fusion partners that facilitate export of the polypeptide when produced recombinantly, fusion partners that facilitate purification of the polypeptide, and fusion partners which enhance the immunogenicity of the polypeptide fragment of the invention are all interesting possibilities. Therefore, the invention also pertains to a fusion polypeptide comprising at least one polypeptide or immunogenic portion defined above and at least one fusion partner. The fusion partner can, in order to enhance immunogenicity, be an-

other polypeptide derived from *M. tuberculosis*, such as of a polypeptide fragment derived from a bacterium belonging to the tuberculosis complex, such as ESAT-6, TB10.4, CFP10, RD1-ORF5, RD1-ORF2, Rv1036, MPB64, MPT64, Ag85A, Ag85B (MPT59), MPB59, , Ag85C, 19kDa lipoprotein, MPT32 and alpha-crystallin, or at least one T-cell
5 epitope of any of the above mentioned antigens ((Skj t et al 2000; Danish Patent application PA 2000 00666; Danish Patent application PA 1999 01020; US patent application 09/0505,739; Rosenkrands *et al* 1998; Nagai et al 1991). The invention also pertains to a fusion polypeptide comprising mutual fusions of two or more of the polypeptides (or immunogenic portions thereof) of the invention.

10

Other fusion partners, which could enhance the immunogenicity of the product, are lymphokines such as IFN- γ , IL-2 and IL-12. In order to facilitate expression and/or purification, the fusion partner can e.g. be a bacterial fimbrial protein, e.g. the pilus components pilin and papA; protein A; the ZZ-peptide (ZZ-fusions are marketed by Pharmacia in Sweden);
15 the maltose binding protein; glutathione S-transferase; β -galactosidase; or poly-histidine. Fusion proteins can be produced recombinantly in a host cell, which could be *E. coli*, and it is a possibility to induce a linker region between the different fusion partners.

Other interesting fusion partners are polypeptides, which are lipidated so that the immunogenic polypeptide is presented in a suitable manner to the immune system. This effect
20 is e.g. known from vaccines based on the *Borrelia burgdorferi* OspA polypeptide as described in e.g. WO 96/40718 A or vaccines based on the *Pseudomonas aeruginosa* OprI lipoprotein (Cote-Sierra J 1998). Another possibility is N-terminal fusion of a known signal sequence and an N-terminal cysteine to the immunogenic polypeptide. Such a fusion results in lipidation of the immunogenic polypeptide at the N-terminal cysteine, when produced in a suitable production host.

Another part of the invention pertains to a vaccine composition comprising a polypeptide (or at least one immunogenic portion thereof) or fusion polypeptide according to the invention. In order to ensure optimum performance of such a vaccine composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant.
30

An effective vaccine, wherein a polypeptide of the invention is recognized by the animal,
35 will in an animal model be able to decrease bacterial load in target organs, prolong sur-

vival times and/or diminish weight loss after challenge with a virulent *Mycobacterium*, compared to non-vaccinated animals.

Suitable carriers are selected from the group consisting of a polymer to which the polypeptide(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the polypeptide(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, ovalbumin or keyhole limpet haemocyanin. Suitable vehicles are selected from the group consisting of a diluent and a suspending agent. The adjuvant is preferably selected from the group consisting of dimethyldioctadecylammonium bromide (DDA), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN- γ , IL-2, IL-12, monophosphoryl lipid A (MPL), Trehalose Dimycolate (TDM), Trehalose Dibehenate and muramyl dipeptide (MDP).

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231 and 4,599,230, all incorporated herein by reference.

Other methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), synthetic polymers of sugars (Carbopol), aggregation of the protein in the vaccine by heat treatment, aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Other possibilities involve the use of immune modulating substances such as cytokines or synthetic IFN- γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

Another interesting possibility for achieving adjuvant effect is to employ the technique described in Gosselin *et al.*, 1992 (which is hereby incorporated by reference herein). In brief, a relevant antigen such as an antigen of the present invention can be conjugated to an antibody (or antigen binding antibody fragment) against the Fc γ receptors on monocytes/macrophages.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection
5 desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 μg to 1000 μg , such as in the range from about 1 μg to 300 μg , and especially in the range from about 10 μg to 50 μg . Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other admini-
10 strations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion,
15 parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

The vaccines are conventionally administered parenterally, by injection, for example, ei-
20 ther subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral for-
25 mulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and advantageously contain 10-95% of active ingredient, preferably 25-70%.

30

In many instances, it will be necessary to have multiple administrations of the vaccine. Especially, vaccines can be administered to prevent an infection with virulent mycobacteria and/or to treat established mycobacterial infection. When administered to prevent an infection, the vaccine is given prophylactically, before definitive clinical signs or symptoms
35 of an infection are present.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune response. The
5 vaccine may comprise two or more polypeptides or immunogenic portions, where all of the polypeptides are as defined above, or some but not all of the peptides may be derived from virulent mycobacteria. In the latter example, the polypeptides not necessarily fulfilling the criteria set forth above for polypeptides may either act due to their own immunogenicity or merely act as adjuvants.

10

The vaccine may comprise 1-20, such as 2-20 or even 3-20 different polypeptides or fusion polypeptides, such as 3-10 different polypeptides or fusion polypeptides.

The invention also pertains to a method for immunising an animal, including a human being, against TB caused by virulent mycobacteria, comprising administering to the animal
15 the polypeptide of the invention, or a vaccine composition of the invention as described above, or a living vaccine described above.

The invention also pertains to a method for producing an immunologic composition according to the invention, the method comprising preparing, synthesising or isolating a polypeptide according to the invention, and solubilizing or dispersing the polypeptide in a medium for a vaccine, and optionally adding other *M. tuberculosis* antigens and/or a carrier, vehicle and/or adjuvant substance.

25 The nucleic acid fragments of the invention may be used for effecting *in vivo* expression of antigens, *i.e.* the nucleic acid fragments may be used in so-called DNA vaccines as reviewed in Ulmer et al 1993, which is included by reference.

Hence, the invention also relates to a vaccine comprising a nucleic acid fragment according to the invention, the vaccine effecting *in vivo* expression of antigen by an animal,
30 including a human being, to whom the vaccine has been administered, the amount of expressed antigen being effective to confer substantially increased resistance to infections caused by virulent mycobacteria in an animal, including a human being.

The efficacy of such a DNA vaccine can possibly be enhanced by administering the gene encoding the expression product together with a DNA fragment encoding a polypeptide which has the capability of modulating an immune response.

- 5 One possibility for effectively activating a cellular immune response for a vaccine can be achieved by expressing the relevant antigen in a vaccine in a non-pathogenic microorganism or virus. Well-known examples of such microorganisms are *Mycobacterium bovis* BCG, *Salmonella* and *Pseudomona* and examples of viruses are Vaccinia Virus and Adenovirus.

10

Therefore, another important aspect of the present invention is an improvement of the living BCG vaccine presently available, wherein one or more copies of a DNA sequence encoding one or more polypeptide as defined above has been incorporated into the genome of the micro-organism in a manner allowing the micro-organism to express and secrete
15 the polypeptide. The incorporation of more than one copy of a nucleotide sequence of the invention is contemplated to enhance the immune response

Another possibility is to integrate the DNA encoding the polypeptide according to the invention in an attenuated virus such as the vaccinia virus or Adenovirus (Rolph et al 1997).

- 20 The recombinant vaccinia virus is able to replicate within the cytoplasm of the infected host cell and the polypeptide of interest can therefore induce an immune response, which is envisioned to induce protection against TB.

The invention also relates to the use of a polypeptide or nucleic acid of the invention for
25 use as therapeutic vaccines as have been described in the literature exemplified by D. Lowry (Lowry et al 1999). Antigens with therapeutic properties may be identified based on their ability to diminish the severity of *M. tuberculosis* infection in experimental animals or prevent reactivation of previous infection, when administered as a vaccine. The composition used for therapeutic vaccines can be prepared as described above for vaccines.

30

The invention also relates to a method of diagnosing TB caused by a virulent mycobacterium in an animal, including a human being, comprising intradermally injecting, in the animal, a polypeptide according to the invention, a positive skin response at the location of injection being indicative of the animal having TB, and a negative skin response at the
35 location of injection being indicative of the animal not having TB.

When diagnosis of previous or ongoing infection with virulent mycobacteria is the aim, a blood sample comprising mononuclear cells (*i.e.* T-lymphocytes) from a patient could be contacted with a sample of one or more polypeptides of the invention. This contacting can
5 be performed *in vitro* and a positive reaction could *e.g.* be proliferation of the T-cells or release of cytokines such as IFN- γ into the extracellular phase. It is also conceivable to contact a serum sample from a subject with a polypeptide of the invention, the demonstration of a binding between antibodies in the serum sample and the polypeptide being indicative of previous or ongoing infection.

10

The invention therefore also relates to an *in vitro* method for diagnosing ongoing or previous sensitisation in an animal or a human being with a virulent mycobacterium, the method comprising providing a blood sample from the animal or human being, and contacting the sample from the animal with the polypeptide of the invention, a significant re-
15 lease into the extracellular phase of at least one cytokine by mononuclear cells in the blood sample being indicative of the animal being sensitised. A positive response being a response more than release from a blood sample derived from a patient without the TB diagnosis plus two standard derivations. The invention also relates to the *in vitro* method for diagnosing ongoing or previous sensitisation in an animal or a human being with a
20 virulent mycobacterium, the method comprising providing a blood sample from the animal or human being, and by contacting the sample from the animal with the polypeptide of the invention demonstrating the presence of antibodies recognizing the polypeptide of the invention in the serum sample.

25 The immunogenic composition used for diagnosing may comprise 1-20, such as 2-20 or even 3-20 different polypeptides or fusion polypeptides, such as 3-10 different polypeptides or fusion polypeptides.

The nucleic acid probes encoding the polypeptide of the invention can be used in a variety
30 of diagnostic assays for detecting the presence of pathogenic organisms in a given sample. A method of determining the presence of mycobacterial nucleic acids in an animal, including a human being, or in a sample, comprising administering a nucleic acid fragment of the invention to the animal or incubating the sample with the nucleic acid fragment of the invention or a nucleic acid fragment complementary thereto, and detecting the pres-
35 ence of hybridised nucleic acids resulting from the incubation (by using the hybridisation

assays which are well-known in the art), is also included in the invention. Such a method of diagnosing TB might involve the use of a composition comprising at least a part of a nucleotide sequence as defined above and detecting the presence of nucleotide sequences in a sample from the animal or human being to be tested which hybridise with
5 the nucleic acid fragment (or a complementary fragment) by the use of PCR technique.

A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide of the invention in an immuno assay, or a specific binding fragment of said antibody, is also a part of the invention. The antibodies can be produced by methods known to the person
10 skilled in the art. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of a polypeptide according to the present invention and, if desired, an adjuvant. The monoclonal antibodies according to the present invention may, for example, be produced by the hybridoma method first described by Kohler and Milstein (1975), or may be produced by recombinant DNA methods such as described in U.S. Pat. No.
15 4,816,567. The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described by McCafferty et al (1990), for example. Methods for producing antibodies are described in the literature, e.g. in US 6,136,958.

A sample of a potentially infected organ may be contacted with such an antibody recognizing a polypeptide of the invention. The demonstration of the reaction by means of
20 methods well known in the art between the sample and the antibody will be indicative of an ongoing infection. It is of course also a possibility to demonstrate the presence of anti-mycobacterial antibodies in serum by contacting a serum sample from a subject with at least one of the polypeptide fragments of the invention and using well-known methods for
25 visualising the reaction between the antibody and antigen.

In diagnostics, an antibody, a nucleic acid fragment and/or a polypeptide of the invention can be used either alone, or as a constituent in a composition. Such compositions are known in the art, and comprise compositions in which the antibody, the nucleic acid fragment or the polypeptide of the invention is coupled, preferably covalently, to at least one
30 other molecule, e.g. a label (e.g. radioactive or fluorescent) or a carrier molecule.

Concordance list

	Protein SEQ ID NO:	DNA SEQ ID NO:	Synonyms
Rv0284	2	1	
Rv0284ct	4	3	
Rv0285	6	5	
Rv0455c	8	7	TB13.7
Rv0569	10	9	TB9.5
Rv1195	12	11	
Rv1386	14	13	
Rv3477	16	15	
Rv3878	18	17	
ORF13A	20	19	
Rv3879c	22	21	
Rv0285-P1	23		
Rv0285-P2	24		
Rv0285-P3	25		
Rv0285-P4	26		
Rv0285-P5	27		
Rv0285-P6	28		
Rv0285-P7	29		
Rv0285-P8	30		
Rv0285-P9	31		
Rv0285-P10	32		
Rv1386-P1	33		
Rv1386-P2	34		
Rv1386-P3	35		
Rv1386-P4	36		
Rv1386-P5	37		
Rv1386-P6	38		
Rv1386-P7	39		
Rv1386-P8	40		
Rv1386-P9	41		
Rv1386-P10	42		
TB9.5-1	43		
TB9.5-2	44		
TB9.5-3	45		
TB9.5-4	46		
TB13.7-1	47		
TB13.7-2	48		
TB13.7-3	49		
TB13.7-4	50		

TB13.7-5	51	
MT3106.1	53	52
Rv0284-P1	54	
Rv0284-P2	55	
Rv0284-P3	56	
Rv0284-P4	57	
Rv0284-P5	58	
Rv0284-P6	59	
Rv0284-P7	60	
Rv0284-P8	61	
Rv0284-P9	62	
Rv0284-P10	63	
Rv0284-P11	64	
Rv0284-P12	65	
Rv0284-P13	66	
Rv0284-P14	67	
Rv0284-P15	68	
Rv0284-P16	69	
Rv0284-P17	70	
Rv0284-P18	71	
Rv0284-P19	72	
Rv0284-P20	73	
Rv0284-P21	74	
Rv0284-P22	75	
Rv0284-P23	76	
Rv0284-P24	77	
Rv0284-P25	78	
Rv0284-P26	79	
Rv0284-P27	80	
Rv0284-P28	81	
Rv0284-P29	82	
Rv0284-P30	83	
Rv0284-P31	84	
Rv0284-P32	85	
Rv0284-P33	86	
Rv0284-P34	87	
Rv0284-P35	88	
Rv0284-P36	89	
Rv0284-P37	90	
Rv0284-P38	91	
Rv0284-P39	92	

Rv0284-P40	93
Rv0284-P41	94
Rv0284-P42	95
Rv0284-P43	96
Rv0284-P44	97
Rv0284-P45	98
Rv0284-P46	99
Rv0284-P47	100
Rv0284-P48	101
Rv0284-P49	102
Rv0284-P50	103
Rv0284-P51	104
Rv0284-P52	105
Rv0284-P53	106
Rv0284-P54	107
Rv0284-P55	108
Rv0284-P56	109
Rv0284-P57	110
Rv0284-P58	111
Rv0284-P59	112
Rv0284-P60	113
Rv0284-P61	114
Rv0284-P62	115
Rv0284-P63	116
Rv0284-P64	117
Rv0284-P65	118
Rv0284-P66	119
Rv0284-P67	120
Rv0284-P68	121
Rv0284-P69	122
Rv3878-P1	123
Rv3878-P2	124
Rv3878-P3	125
Rv3878-P4	126
Rv3878-P5	127
Rv3878-P6	128
Rv3878-P7	129
Rv3878-P8	130
Rv3878-P9	131
Rv3878-P10	132
Rv3878-P11	133

Rv3878-P12	134	
Rv3878-P13	135	
Rv3878-P14	136	
Rv3878-P15	137	
Rv3878-P16	138	
Rv3878-P17	139	
Rv3878-P18	140	
Rv3878-P19	141	
Rv3878-P20	142	
Rv3878-P21	143	
Rv3878-P22	144	
Rv3878-P23	145	
MT3106.1-p1	146	
MT3106.1-p2	147	
MT3106.1-p3	148	
MT3106.1-p4	149	
MT3106.1-p5	150	
MT3106.1-p6	151	
MT3106.1-p7	152	
MT3106.1-p8	153	
MT3106.1-p9	154	
MT3106.1-p10	155	
MT3106.1-p11	156	
Rv0284-F		157
Rv0284-R		158
Rv0285-F		159
Rv0285-R		160
Rv3878-F		161
Rv3878-R		162
ORF13A-F		163
ORF13A-R		164
Rv1195-F		165
Rv1195-R		166
Rv1386-F		167
Rv1386-R		168
Rv3477-F		169
Rv3477-R		170
TB9.5 15AA from sequencing	171	
TB13.7 15AA from sequencing	172	

Legends to figures

Figure 1: Stimulation of IFN- γ production by synthetic peptides in PBMC from PPD positive healthy donors. Single peptides were tested at concentrations of 10 μ g, 5 μ g and 2.5 μ g/ml in 200 μ l of cell culture. Pools of peptides were tested at 1 μ g, 0.5 μ g and 0.25 μ g/ml of each peptide. Results are presented as pg IFN- γ /ml of the maximum stimulation. Recombinant antigens were included for comparison.

Figure 2A: The antibody response of 48 TB patients to ORF13A evaluated by ELISA. The OD indicated is the mean of two wells coated with 1 μ g/ml ORF13A and the serum is diluted 1:100 in PBS.

Figure 2B: The antibody response of 15 BCG vaccinated healthy donors to ORF13A evaluated by ELISA. The OD indicated is the mean of two wells coated with 1 μ g/ml ORF13A and the serum is diluted 1:100 in PBS.

Figure 2C: The antibody response of 19 non BCG-vaccinated healthy donors to ORF13A evaluated by ELISA. The OD indicated is the mean of two wells coated with 1 μ g/ml ORF13A and the serum is diluted 1:100 in PBS.

Figure 3: Stimulation of T-cell proliferation by synthetic peptides derived from Rv3878. T-cell lines against STCF were derived from PBMC isolated from PPD positive donors. Peptides were tested at 10 μ g and 5 μ g/ml. Results are presented as cpm of the maximum stimulation. n.d = not determined.

Examples

Example 1: Cloning and expression of Rv0284, Rv0285, Rv3878, Rv1195, Rv1386, Rv3477 and ORF13A

The coding region of Rv0285, Rv3878, the 3'-part (380 bp) of Rv0284 and 5'-part of ORF13A (543 bp of Rv3879c) were amplified by PCR using following primer sets:

Rv0284-F: CTG AGA TCT CAG GTA CCG GAT TCG CCG
BglII

Rv0284-R: CTC CCA TGG TCA TGA CTG ACT CCC CTT
NcoI

Rv0285-F: CTG AGA TCT ATG ACG TTG CGA GTG GTT
BglII

5 **Rv0285-R:** CTC CCA TGG TCA GCC GCC CAC GAC CCC
NcoI

Rv3878-F: CTG AGA TCT GCT ACT GTT AAC AGA TCG
BglII

10

Rv3878-R: CCG CTC GAG CTA CAA CGT TGT GGT TGT
XhoI

15 **ORF13A-F:** CCC AAG CTT ATG AGT ATT ACC AGG CCG
HindIII

ORF13A-R: CTC CCA TGG TCA CGA CTT CTG CTG AAG CAA
NcoI

20

PCR reactions contained 10 ng of *M. tuberculosis* H37Rv DNA in 1x low salt Taq⁺ buffer from Stratagene supplemented with 250 μ M of each of the four nucleotides (Boehringer Mannheim), 0.5 mg/ml BSA (IgG technology), 1% DMSO (Merck), 5 pmoles of each primer and 0.5 unit Taq⁺ DNA polymerase (Stratagene) in 10 μ l reaction volume. Reactions were initially heated to 94°C for 15 sec, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec, and finally by 72°C for 5 min.

The PCR fragments were cloned into the TA cloning vector pCR2.1 (Invitrogen) and then transferred to the pMCT3 expression vector at the restriction sites indicated by the primers above. The coding regions of Rv1195, Rv1386 and Rv3477 were amplified by PCR using the following primer sets:

Rv1195-F: gggg ACA AgT TTg TAc AAA AAA gCA ggC TTA gTgTCTTTCgTgATggCATACC

Rv1195-R: gggg AC CAC TTT gTA CAA gAA AgC Tgg gTC CTA TTAgtCTggCCgCCgC

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Rv1386-F: gggg ACA AgT TTg TAc AAA AAA gCA ggC TTA gTgACgTTgCgAgTCgTTCC

Rv1386-R: gggg AC CAC TTT gTA CAA gAA AgC Tgg gTC CTA TAgtCCCACCgCTgAgATACg

Rv3477-F: gggg ACA AgT TTg TAc AAA AAA gCA ggC TTA gTgTCTTTCACTgCgCAACCg

40 Rv3477-R: gggg AC CAC TTT gTA CAA gAA AgC Tgg gTC CTA gCCggTgACCACAgCgTT

PCR reactions were carried out by Platinum[®] Tag DNA Polymerase (GIBCOBRL[®]) in 50 µl reaction volume containing 60 mM Tris-SO₄ (pH 8.9), 18 mM Ammonium Sulfate, 0.2 mM of each of the four nucleotides, 0.2 µM of each primer and 10 ng of *M. tuberculosis* H37Rv DNA. The reaction mixtures were initially heated to 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 60°C for 45 sec and 72°C for 2 min, and finally by 72°C for 15 min. The PCR products were precipitated by PEG/MgCl₂, and then dissolved in 50 µl of TE buffer. DNA fragments were then cloned and expressed in Gateway[™] Cloning system (GIBCOBRL[®]). First, to create Entry Clones, 5 µl of each DNA fragment was mixed with 1 µl of pDONR201, 2 µl of BP CLONASE Enzyme Mix and 2 µl of BP Reaction Buffer. The recombination reactions were carried out at 25°C for 60 min. After degrading the Enzymes by Proteinase K at 37°C for 10 min, 5 µl of each sample was used to transform *E. coli* DH5α competent cells. The transformants were selected on LB plates containing 50 µg/ml kanamycin. Second, to create Expression clones, 2 µl of each Entry Clone DNA was mixed with 1 µl of the expression vector, pDest17, 2 µl LR reaction buffer and 2 µl LR CLONASE Enzyme Mix in a total volume of 10 µl. After the recombination reaction at 25°C for 60 min and proteinase K treatment at 37°C for 10 min, 5 µl of the samples were used to transform *E. coli* BL21-SI competent cells. The transformants were selected on LBON (LB without NaCl) plates containing 100 µg/ml ampicillin. The resulting recombinant antigens carried 6-histidine residues at the N-terminal. All clones were confirmed by DNA sequencing.

To express his-tagged recombinant antigens in pMCT3 vector, 100 ml of an overnight culture of XL-1 blue carrying the plasmid construct was added to 900 ml of LB-media containing 100 µg/ml ampicillin, grown at 37°C with shaking. 1 mM IPTG was added at OD₆₀₀ = 0.4-0.6 and the culture was incubated for additional 3 - 16 hours before harvesting of cells.

To express his-tagged recombinant antigens in pDest17, BL21-SI cells were cultured in LBON medium at 30°C and the induction of recombinant antigen synthesis was achieved by adding 0.3 M NaCl to the medium at OD₆₀₀ = 0.4-0.6, and cells were harvested 3 hours later.

For purification, the cell pellet was resuspended in 20 ml of Sonication buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 10% Glycerol, 5 mM β-ME, 0.01% Tween 20 and 1 mM imida-

zole). Cells were lysed and DNA was digested by treating with lysozyme (0.1 mg/ml) and DNase I (2.5 µg/ml) at room temperature for 20 min with gentle agitation. The recombinant protein was brought to solution by adding 80 ml of Sonication Buffer containing 8 M urea and sonicated the sample 5 x 30 sec, with 30 sec pausing between the pulses.

- 5 After centrifugation, the lysate was applied to a 5 ml TALON column (Clontech). The column was then washed with 25 ml of urea containing Sonication buffer, and the bound protein was eluted by imidazole steps (5, 10, 20, 40 and 100 mM) in the same buffer. The fractions were analyzed by silver stained SDS-PAGE, and recombinant protein containing fractions were pooled. Further purifications were achieved either by anion- and cation-
- 10 exchange chromatography on Hitrap columns (Pharmacia, Uppsala, Sweden) or by electroelution as described below: The pooled TALON fractions were dialyzed against 3 x 1 L of 10 mM Tris-Cl (pH 8.0), 0.15 M NaCl and 0.1% SDS. Two mg of TALON purified recombinant antigen was subjected to SDS-PAGE on a 16 x 16 cm gel. After separation, the recombinant antigen band was cut out and the protein was eluted by a Model 422
- 15 Electro-Eluter (Bio-Rad). SDS was removed from eluted protein by Chloroform/Methanol extraction.

Example 2: Biological activity of the recombinant antigens.

- The purified recombinant proteins were screened for the ability to induce a T cell response measured as IFN-γ release and/or cell proliferation. A preliminary screening involved testing of the IFN-γ induction and/or cell proliferation of T cell lines generated from PPD positive donors. This test was followed by measuring the response in PBMC preparations obtained from TB patients, PPD positive as well as negative healthy donors.
- 20

Interferon-γ induction and cell proliferation of T cell lines:

- 25 **Human donors:** PBMC were obtained from healthy donors with a positive *in vitro* response to PPD.

- T cell line preparation:** T cell lines were prepared by culturing 5×10^6 freshly isolated PBMC/ml with viable *M. tuberculosis* at a ratio of 5 bacteria per macrophage in a total volume of 1 ml. The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, N.Y) supplemented with HEPES, and 10% heat-inactivated NHS. After 7 days in culture at 37°C and 5% CO₂, T cells were supplemented with 50 U/ml of r-IL-2 (Boehringer Mannheim) for approximately 7 days. Finally, in one experiment (Table 1), the T cell lines were
- 30

tested for reactivity against the recombinant antigens by stimulating $1-5 \times 10^5$ cells/ml with 5 μ g/ml of PPD, 3 μ g/ml of rRv0284ct (C-terminal part), 5 μ g/ml of rRv0285, and 2.5 μ g/ml of rRv3878 in the presence of 5×10^5 autologous antigen-presenting cells/ml. In another experiment (Table 1a), T cells were stimulated with 5 μ g/ml and 1 μ g/ml of each recombinant antigen indicated in the table. No ag and PHA were used as negative and positive controls, respectively. The supernatants were harvested after 4 days of culture and stored at -80°C until the presence of IFN- γ were analysed.

Cytokine analysis: Interferon- γ (IFN- γ) was detected with a standard sandwich ELISA technique using a commercially available pair of monoclonal antibodies (Endogen, MA, US) and used according to the manufacturer's instructions. Recombinant IFN- γ (Endogen, MA, US) was used as a standard. All data are means of duplicate wells and the variation between the wells did not exceed 10 % of the mean. Responses obtained with five T cell lines are shown in Table 1 and Table 1a.

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T-cell proliferation assays: After removal of supernatant for IFN- γ assays, 0.5 μ Ci of [methyl- ^3H]thymidine were added to the same wells supplemented with 10% NHS in RPMI for another 16-20 hours. The cells were thereafter harvested with a Skatron cell harvester onto filter mats, dried, and immersed in scintillation fluid before reading the incorporation of thymidine on a beta liquid scintillation counter (Wallac). Results from 3 T cell lines are shown in Table 1b.

20

As shown in Table 1, high levels of IFN- γ release are observed after stimulation with the recombinant antigens ranging from 33% (rRv0284ct) to 83% (rRv3878) of the response seen after stimulation with PPD. The antigenicity of the recombinant antigens was confirmed by three additional T-cell lines as shown in Table 1a and Table 1b.

Table 1. Stimulation of two T cell lines with recombinant rRv0284ct, rRv0285, and rRv3878. Responses to PHA and PPD are shown for comparison. Results are presented as pg IFN- γ /ml.

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T cell line						
Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	rRv0284ct (3 μ g/ml)	rRv0285 (5 μ g/ml)	rRv3878 (2.5 μ g/ml)
1	50	2975	2742	914	2019	1072
2	50	1482	803	352	548	667

Table 1a. Stimulation of three T cell lines with rRv0285 and rRv3878. Responses to PHA and PPD are shown for comparison. Results are presented as pg IFN- γ /ml of the maximum stimulation in the presence of either 5 μ g/ml or 1 μ g/ml of recombinant antigens.

T cell line

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	rRv0285	rRv3878
3	136	4467	2425	1189	504
4	2	1996	1175	626	413
5	4	5410	4490	2804	2034

5

Table 1b. Stimulation of T cell proliferation by rRv0285 and rRv3878. Results are presented as Stimulation Index (SI). The maximum stimulation in the presence of either 5 μ g/ml or 1 μ g/ml of recombinant antigens is given.

Donor	rRv0285	rRv3878
3	8.4	N.D
4	5.8	4.3
5	31.3	16.1

10

Interferon- γ release from PBMC isolated from human TB patients and PPD positive and negative healthy donors

Human donors: PBMC were obtained from healthy donors with a positive *in vitro* response to purified protein derivative (PPD) or non-vaccinated healthy donors with a negative *in vitro* response to PPD. PBMC were also obtained from TB patients with microscopy or culture proven infection. Blood samples were drawn from TB patients 0-6 months after diagnosis.

Lymphocyte preparations and cell culture: PBMC were freshly isolated by gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway) and stored in liquid nitrogen until use. The cells were resuspended in complete RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 1% penicillin/streptomycin (Gibco BRL, Life Technologies), 1% non-essential-amino acids (FLOW, ICN Biomedicals, CA, USA), and 10% heat-inactivated normal human AB serum (NHS). The viability and number of the cells were determined by Nigrosin staining. Cell cultures were established with 1.25×10^5 PBMCs in 100 μ l in microtitre plates (Nunc, Roskilde, Denmark) and stimulated with 5 μ g/ml PPD or rRv0284ct and rRv3878 in a final concentration of 2.5 and 5 μ g/ml, respectively; or with 2.5 and 10 μ g/ml of rRv0285, Rv1195, rRv1386 and Rv3477. No antigen (No ag) was used as a negative control, whereas phytohaemagglutinin (PHA) was

used as a positive control. Moreover, the response to a well-known TB-specific protein, ESAT-6, was included for comparison. Supernatants for the analysis of secreted cytokines were harvested after 5 days of culture, pooled, and stored at -80 °C until use.

- 5 **Cytokine analysis:** IFN- γ was detected as above. Responses obtained with PBMCs from 14 individual donors are shown in Table 2.

As shown in Table 2, stimulation of PBMC from TB patients as well as PPD positive donors with rRv0284ct and rRv3878 resulted in a marked release of IFN- γ with 55% of the
10 donors recognizing the recombinant antigens at a level of more than 500 pg/ml. As expected, none of the recombinant antigens gave rise to IFN- γ release in PPD negative donors. The effects of stimulating with rRv0285, rRv1386, rRv1195 and rRv3477 on IFN- γ release in PBMC are demonstrated in Table 2a.

Table 2. Stimulation of PBMCs from 4 TB patients, 7 PPD positive healthy donors, and 3 PPD negative healthy donors with recombinant antigen. Responses to PHA, PPD, and ESAT6 are shown for comparison. Results are given as pg IFN- γ /ml.

TB patients

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	ESAT-6 (5 μ g/ml)	rRv0284ct (2.5 μ g/ml)	rRv3878 (5 μ g/ml)
1	3	4541	4074	2154	809	3
2	92	3408	4891	611	236	2029
3	5	5282	4647	2827	308	149
4	10	4531	2077	38	140	287

5

PPD positive healthy donors

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	ESAT-6 (5 μ g/ml)	rRv0284ct (2.5 μ g/ml)	rRv3878 (5 μ g/ml)
1	74	5413	3339	0	382	77
2	14	5614	3852	198	1324	633
3	7	6165	5808	4	2951	2732
4	63	6532	6314	1567	3009	3482
5	43	4733	6195	1272	5166	2589
6	5	3809	2582	15	5	71
7	31	6716	2275	424	1449	832

PPD negative healthy donors

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	ESAT-6 (5 μ g/ml)	rRv0284ct (2.5 μ g/ml)	rRV3878 (5 μ g/ml)
1	0	3354	113	0	269	17
2	0	3803	563	0	22	0
3	0	3446	525	10	203	34

10

Table 2a. Stimulation of IFN- γ production by rRv0285, rRv1386, rRv1195 and rRv3477 in PBMC from PPD negative controls, PPD positive healthy donors as well as TB patients. TB10.4 was included for comparison.

Donor	No ag	PPD	Rv0285	Rv1386	Rv1195	Rv3477	TB10.4
PPD negative healthy donors ¹⁾							
K150	12	265	0	5	2	3	0
K151	22	50	0	nd	nd	nd	10
K156	17	522	0	166	86	71	2
K159	27	155	1	16	12	19	3
K160	16	242	6	62	9	26	4
K161	35	510	2	40	23	33	0
K162	31	352	89	71	nd	0	9
TB-patients							
98-160	5	>5549	nd	2885	nd	nd	nd
99-203	0	2232	914	nd	nd	nd	903
99-208	2	4098	317	186	nd	11	8
00-199	11	2592	456	nd	nd	nd	3116
00-211	0	10633	2533	2862	1814	1243	4161
00-217	22	4140	124	57	nd	66	535
00-218	0	1578	21	28	nd	13	38
00-220	18	9476	77	106	437	34	3063
00-222	28	9824	2226	1071	226	44	3600
00-223	89	10412	2458	nd	nd	nd	4537
PPD positive healthy donors							
K119	0	7464	227	296	nd	111	585
K131	29	1730	1777	20	17	31	7
K147	86	4520	18	79	47	26	12
K148	52	8293	78	11	86	58	3843
K149	72	12730	932	243	nd	489	38
K152	96	6120	0	946	40	517	1303
K153	5	12391	2	467	nd	622	709
K155	5	9397	0	9	15	37	973
K167	105	15770	3531	1811	nd	nd	4881
k172	10	18811	21420	4	3717	10	110
K174	3	1443	492	44	56	17	160
KTb1	34	13748	3067	1307	nd	nd	9431
KTb2	23	8104*	391	nd	nd	nd	2237
KTb10	4	2394*	292	nd	nd	nd	46
L	46	7832*	949	nd	nd	nd	349
C	33	6538	303	3	255	116	5

¹⁾ IFN- γ median=13294 pg/ml on stimulation with PHA . * IFN- γ on stimulation with STCF

BMC from 6 additional TB patients were obtained, and the T-cell stimulatory effect of rRv1195 was also tested in these PBMCs. The results are shown in Table 2b.

Table 2b Stimulation of IFN- γ production by rRv1195 in PBMCs from six TB patients.

Donor	No ag	PPD	Rv1195
97-83	42	>3531	1060
97-138	13	>3366	231
98-149	256	>3449	2855
99-163	45	>2303	422
01-226	68	>3994	2133
PT36	342	1510	411

Together, these analyses using PBMC and T cell lines, respectively, indicate that rRv0284ct, rRv0285, rRv1386 and rRv3878 are highly biologically active and frequently
 5 recognized by PPD positive donors and TB patients. Though less frequently recognized by these donors rRv1195 and rRv3477 are additionally highly biologically active.

As is expected, due to the genetical heterogeneity of the human population some of the recombinant antigens are recognized more frequently and to a higher level than others are.

10

Skin test reaction in TB infected guinea pigs

The skin test reactivity of the recombinant antigens was tested in *M. tuberculosis* infected guinea pigs. A group of 5 female outbred guinea pigs of the Dunkin Hartley strains (Møl-
 15 legaard Breeding and Research Center A/S, Lille Skensved, Denmark) were infected by the aerosol route in an exposure chamber of a Glas-Col® Inhalation Exposure System, which was calibrated to deliver approximately 20-25 *M. tuberculosis* Erdman bacilli into the lungs of each animal. As a control, the skin test reactivity of uninfected guinea pigs was tested. Skin tests were performed 28 days after infection with injection of 5 μ g of
 20 rRv0284ct, rRv0285, and rRv3878. As a positive control, the guinea pigs were sensitised with 10 tuberculin units (TU) of PPD (1TU = 0.02 μ g) whereas injection of phosphate-buffered saline (PBS) was used as a negative control. Skin test responses (diameter of erythema) were read 24 h later by two experienced examiners and the results were expressed as the mean of the two readings. The variation between the two readings was
 25 less than 10%. Skin test responses larger than 5 mm were regarded as positive.

As seen in Table 3, injection of rRv3878 induced a marked Delayed Type Hypersensitivity (DTH) reaction at the same level as after injection with PPD. rRv0284ct and rRv0285 resulted in a highly significant DTH reaction ($P < 0.005$; Tukey test). As expected, none of
 30 the antigens induced non-specific response in uninfected guinea pigs (Table 4).

Table 3. DTH erythema diameter (shown in mm) in guinea pigs aerosol infected with *M. tuberculosis* after stimulation with recombinant antigens.

Antigen ^a	Skin reaction (mm) ^b	SEM
PBS	3.10	0.30
PPD	13.10	1.18
rRv0284ct	8.40	0.45
rRv0285	7.00	1.08
rRv3878	14.56	1.05

^a The recombinant antigens were tested in a concentration of 5 µg, whereas 10 TU of PPD were used.

^b The skin reactions are measured in mm erythema 24 h after intradermal injection. The values are the mean of erythema diameter of five animals and the SEM are indicated. The values for rRv3878 are the mean of four animals.

Table 4. DTH erythema diameter (shown in mm) in non-infected guinea pigs after stimulation with recombinant antigens.

Antigen ^a	Skin reaction (mm) ^b	SEM
PBS	2.60	0.36
PPD	3.00	0.44
rRv0284ct	2.5	0.18
rRv0285	3.45	0.74
rRv3878	2.5	0.18

^a The recombinant antigens were tested in a concentration of 5 µg, whereas 10 TU of PPD were used.

^b The skin reactions are measured in mm erythema 24 h after intradermal injection. The values are the mean of erythema diameter of five animals and the SEM are indicated.

Example 3: Immunological response to synthetic polypeptides

5

Peptide synthesis: Ten overlapping peptides to Rv0285 and Rv1386 respectively, were synthesized. Synthetic polypeptides were purchased from Mimotopes Pty Ltd. The peptides were synthesized by Fmoc solid phase strategy. No purification steps were performed. Lyophilised peptides were stored dry until use.

10

Rv0285 peptides:

	Rv0285-P1	TLRVPEGLAAASAAVEA
	Rv0285-P2	ASAAVEALTARLAAHAS
15	Rv0285-P3	TARLAAHASAAPVITAV
	Rv0285-P4	AAPVITAVPPAADPVSL
	Rv0285-P5	PAADPVSLQTAAGFSAQG
	Rv0285-P6	AAGFSAQGVHAAVTAEG
	Rv0285-P7	HAAVTAEGVEELGRAGVG
20	Rv0285-P8	GVEELGRAGVGVGEGAS
	Rv0285-P9	GVGEGASYLAGDAAAAA
	Rv0285-P10	SYLAGDAAAAATYGVVGG

Rv1386 peptides:

	Rv1386-P1	TLRVVPESLAGASAAIEA
5	Rv1386-P2	ASAAIEAVTARLAAAHAA
	Rv1386-P3	TARLAAAHAAAAPFIAAV
	Rv1386-P4	AAPFIAAVIPPGSDSVSV
	Rv1386-P5	PGSDSVSVCNAVEFSVHG
	Rv1386-P6	AVEFSVHGSQHVAMAAQG
10	Rv1386-P7	HVAMAAQGVEELGRSGVG
	Rv1386-P8	GVEELGRSGVGVAESGAS
	Rv1386-P9	GVAESGASYAARDALAAA
	Rv1386-P10	SYAARDALAAASYLSGGL

- 15 **PBMC culture and IFN- γ assay:** PBMC were isolated and cultured as described in Example 2. Single peptides were tested at concentrations of 10 μ g, 5 μ g and 2.5 μ g/ml in 200 μ l of cell culture. Pools of peptides were tested at 1 μ g, 0.5 μ g and 0.25 μ g/ml of each peptide. IFN- γ levels were measured by the method described in Example 2.

20 **PBMC recognition of peptides from Rv0285 and Rv1386**

The ability of these peptides to induce IFN- γ production in PBMC was assayed. The results from three PPD positive healthy donors (referred to as KTB1, KTB10 and K172, respectively) are shown in Fig.1. The pools of peptides from Rv0285 (referred to as Rv0285 p1 – Rv0285 p10) stimulated IFN- γ production in PBMC from all three donors. This is

25 consistent with the results obtained with recombinant Rv0285 (Table 2a and Fig.1). When tested singly, seven peptides were recognized by the three donors, indicating the presence of multiple immunogenic portions scattered through out the protein sequence of Rv0285.

30 The pools of peptides from Rv1386 and recombinant Rv1386 stimulated IFN- γ production in PBMC from two of the three donors. Four of the peptides were also positive when tested as single peptides. The synthetic peptides were also tested in PBMC from two PPD negative controls; as expected, no stimulation of IFN- γ production was detected for these donors (results not shown).

35

Example 3a: PBMC recognition of peptides derived from MT3106.1

A BLAST-P search of the GMT.pep database at TIGR CMR revealed an open reading frame which is highly related to Rv0285. This ORF is designated MT3106.1, and the pre-

dicted initiation codon is 33 codons upstream of the corresponding initiation codon in Rv0285. Amino acid sequence alignment revealed that the Rv0285-corresponding part of MT3106.1 has 80% sequence identity to the former, and a peptide fragment spanning residues 2 –29 on Rv0285 is 100% conserved on Mt3106.1. This segment of peptide
 5 contains at least 2 distinct T-cell epitopes as demonstrated by the results in Fig. 1 (Rv0285-p1 and Rv0285-p2, respectively). Eleven additional overlapping peptides of MT3106.1 (MT3106.1-p1 - MT3106.1-p11, SEQ ID NO 146-156) were synthesized and analyzed for their ability to induce IFN- γ production in PBMCs from donor K172. Peptide MT3106.1-p7 was highly reactive and stimulated IFN- γ production to a level of 12079
 10 pg/ml, which corresponds to 87% of the activity obtained with PPD.

PBMC from 6 additional TB patients were obtained, and the T-cell stimulatory effect of rRv1195 was also tested in these PBMCs. The results are shown in Table 2b.

15 **Example 3b. Recognition of synthetic peptides by T-cell lines derived from PBMC of PPD positive subjects.**

Non-overlapping peptides (Rv0284-p1 - Rv0284-p69, SEQ ID NO 54-122) were synthesized for the part of Rv0284 that was not included in rRv0284ct. Peptides were tested as
 20 pools consisting of 2 or 3 peptides each. T-cell stimulatory effects were seen in a number of peptide pools. The largest effects on stimulation of IFN- γ release were obtained with peptide pools containing Rv0284-p3, Rv0284-p4, Rv0284-p7, Rv0284-p8, Rv0284-p9, Rv0284-p13, Rv0284-p17, Rv0284-p18, Rv0284-p19, Rv0284-p27, Rv0284-p37, Rv0284-p41, Rv0284-p42, Rv0284-p43, Rv0284-p47, Rv0284-p50, Rv0284-p51;
 25 Rv0284-p52, and Rv0284-p53.

Twenty-three overlapping peptides were synthesised for Rv3878 (Rv3878-p1 - Rv3878-p23, SEQ ID NO 123-145). An initial screening of the peptides in four T-cell lines revealed a number of T-cell epitopes (Fig. 3).

30

Example 4: Identification of TB9.5 and TB13.7

Short-time culture filtrate (ST-CF) was produced from living *Mycobacterium tuberculosis* as previously described and used as an antigen source (Andersen, P. et al 1991). In brief,
 35 ST-CF was produced by growing *M. tuberculosis* H37Rv (4×10^6 CFU/ml) on modified

Sauton medium in an incubator at 37 °C at gentle agitation for 7 days. The culture supernatant was steril-filtered and concentrated on a Amicon YM3 membrane. The culture filtrate was hereafter precipitation with 80 % ammonium sulphate and the precipitated proteins were removed by centrifugation and after washing resuspended in buffer containing

5 8 M urea, CHAPS 0.5% (w/v) and 5% glycerol. 250 mg of protein was separated on the Rotofor Isoelectrical Cell (Bio-Rad) in a pH gradient with 3% Biolyt 3/5 and 1% Biolyt 4/6. Fraction 3-8 were pooled, concentrated and buffer exchanged to PBS on a Centriprep concentrator with a 3 kDa cut off membrane. 100 ug of protein as separated by two-dimensional electrophoresis by applying the sample on immobilized pH 4-7 linear gradient

10 13 cm strips (Amersham Pharmacia Biotech) and the focusing was performed at 500 V for 1 hour, 1000 V at 1 hour followed by 2 hours at 8000 V in a IPGphor unit. The second dimension was performed in 10-20% SDS-PAGE gradient gels in the protean IIxi system (Bio-Rad). The proteins were transferred to a PVDF membrane which was stained for by Coomassie brilliant Blue and two spots was excised and subjected to N-terminal se-

15 quencing analysis by automated Edman degradation using a Procise 494 sequencer (Applied Biosystems) as described by the manufacturer.

Sequence analysis and peptide synthesis

The two spots were named TB9.5 and TB13.7. For each of the two protein spots a sequence of 15 amino acids was obtained.

20 For TB9.5: MKAKVGDILVIKGAT (SEQ ID NO 171)

For TB13.7: DSTEDFPIPXRMXAT (SEQ ID NO 172)

"X" denotes an amino acid, which could not be determined.

The two sequences were used for a homology search using the BLAST program on the

25 *M. tuberculosis* database: <http://genolist.pasteur.fr/TubercuList/>. For TB9.5 the 15 determined amino acids was 100% identical to the sequence of Rv0569, which is an 88 amino acids long protein. For TB13.7 the 13 determined amino acids was 100% identical to the sequence of Rv0455c. The 13 N-terminally determined amino acids starts at amino acids 31 in the predicted sequence of Rv0455c, indication the presence of a signal peptide,

30 which has been cleaved off. This is in agreement with the prediction of a signal peptide in Rv0455c by database analysis of the amino acids sequence using the program Signal P at <http://www.cbs.dtu.dk/services/SignalP/>, which also predicts the most likely cleavage site between position 30 and 31.

Overlapping peptides was produced for the mature version of each of the two proteins by Schafer-N, Copenhagen, Denmark as indicated below. The peptides were synthesized on polyamide resins using Fmoc-strategy and purified by reverse phase HPLC on C18-
 5 columns in water/acetonitrile gradients containing 0.1%TFA (trifluoroacetic acid). Purified peptides were lyophilized and stored dry until reconstitution in PBS.

TB9.5-1: MKAKVGDWLVIKGATIDQPDHRGLIIEVRS

TB9.5-2: HRGLIIEVRSSDGSPPYVVRWLETDHVATV

10 TB9.5-3: VRWLETDHVATVIPGPDVAVVTAAEQNAAD

TB9.5-4: VTAAEQNAADERAQHRFGAVQSAILHARGT

TB13.7-1: DSTEDFPIPRRMIATTCDAEQYLA AVRDT S

TB13.7-2: QYLA AVRDT SPVYYQRYMIDFNNHANLQQA

15 TB13.7-3: FNNHANLQQATINKAHWFFSLSPAERRDYS

TB13.7-4: LSPAERRDYSEHFYNGDPLTFAWVNHMKIF

TB13.7-5: FAWVNHMKIFFNNKGVVAKGTEVCNGY

Immunological activity of TB9.5 and TB13.7

The immunological relevance of the peptides in TB patients was tested by analysing the
 20 ability of the peptides to induce an IFN- γ production or a cell proliferation on PBMC isolated from human TB patients and PPD negative healthy controls (table 5 and table 7).

The TB9.5 peptides were in addition tested for ability to induce IFN- γ and cell proliferation on T cell lines generated from TB patients driven by ST-CF or *M. tuberculosis* sonicate (table 6). Lymphocyte preparation and T-cell lines generation were performed as de-
 25 scribed in example 2.

Table 5: Stimulation of PBMC from three TB patients and three PPD negative healthy controls with pools of synthetic peptides from TB9.5 and TB13.7 in total of 10 ug/ml. 2.5 ug/ml of each peptide TB9.5-1, TB9.5-2, TB9.5-3 and TB9.5-4 were pooled and tested as TB9.5. 2 ug/ml of each peptide TB13.7-1, TB13.7-2, TB13.7-3, TB13.7-4 and TB13.7-5 were pooled and tested as TB13.7. The response to 5 ug/ml ST-CF is shown for comparison. Results are presented as pg IFN- γ /ml.

	TB patients			Healthy controls		
Antigen	PT1	PT2	PT3	H1	H2	H3
Control	0	0	0	9	10	0
ST-CF	4803	11810	3221	28	10	0
TB9.5 10ug/ml	38	59	479	39	0	2
TB9.5 2.5ug/ml	37	56	115	9	7	40
TB13.7 10ug/ml	160	36	29	5	15	13
TB13.7 2.5ug/ml	131	54	70	15	0	0

Pools of the peptides are tested on PBMC purified from human TB patients and healthy controls as seen in table 5. The pools of peptides from TB9.5 were recognized more frequently by TB patients than by the healthy controls. This demonstrates that a positive response is specific for TB patients. TB13.7 was also recognized more frequently by the 5 tested TB patients compared to the healthy controls. It is to be expected that not all of the patients recognized each of the peptides pools, due to the genetically heterogeneity of the human population.

10 Interestingly, it was not the same patient recognizing the two peptide pools indication that the use of a combination of two peptide pools could be superior compared to using the single peptide pools.

The peptides from TB9.5 was in addition tested for ability to induce an IFN- γ response or cell proliferation on five T cell lines derived from TB patients (table 6). TB9.5-1 was positive in most of the tested T-cell lines demonstrating the presence of one or more broadly 15 recognized T cell epitope within this sequence (table 6). Furthermore, TB9.5-2, TB9.5-3 and T9.5-4 were positive in at least one out of the five T cell lines tested demonstrating that these sequences also contains at least one T cell epitope. The presence of multiple

epitopes in the TB9.5 protein makes the full-length protein or peptides derived hereof an attractive candidate for a TB vaccine.

Table 6: Stimulation of five T cell lines derived from TB patients with synthetic overlapping peptides from TB9.5. Results are presented as pg IFN- γ /ml and cell proliferation. The peptides are tested in 1ug/ml and 10ug/ml and results are shown for the concentration given the highest response. The response to 5 μ g/ml ST-CF is shown for comparison.

Antigen	T-cell line 1		T-cell line 2		T-cell line 3		T-cell line 4		T-cell line 5	
	IFN- γ	CPM	IFN- γ	CPM	IFN- γ	CPM	IFN- γ	CPM	IFN- γ	CPM
Control	133	1359	0	184	120	397	62	2550	9	333
ST-CF	4581	26296	3552	21239	2748	12118	2860	18624	4294	29736
TB9.5-1	1438	9116	407	3987	512	1749	42	2033	17	1252
TB9.5-2	3	919	341	3395	69	606	20	1718	10	322
TB9.5-3	26	1145	120	1859	88	537	49	2410	1	331
TB9.5-4	86	2556	519	3887	219	839	28	2860	3	1036
TB9.5-pool	208	3544	52	1825	127	831	6	1738	2	626

Table 7: Stimulation of PBMCs from two TB patients and two healthy controls with synthetic peptides from the TB13.7 protein. Responses to PPD are given for comparison. Control is stimulation without antigen. Results are given as pg IFN- γ /ml

Antigen/ conc.		TB patients		Healthy controls	
Control		PT1	PT2	H1	H2
PPD	5 ug/ml	5549	1269	1570	11
13.7-1	10 ug/ml	20	2	26	42
13.7-1	2.5 ug/ml	6	1	23	47
13.7-2	10 ug/ml	7	2	21	55
13.7-2	2.5 ug/ml	5	3	21	49
13.7-3	10 ug/ml	11	4	20	54
13.7-3	2.5 ug/ml	10	2	28	45
13.7-4	10 ug/ml	8	7	15	24
13.7-4	2.5 ug/ml	8	6	16	30
13.7-5	10 ug/ml	648	5	18	27
13.7-5	2.5 ug/ml	205	7	22	29

5

The 13.7 peptides were tested on PBMC isolated from two TB patients and two healthy controls. As seen in table 7 one of the two TB patients recognized peptide TB13.7-5 while

no of the healthy controls recognized any of the peptides tested. This demonstrates that an epitope is present in peptide TB13.7-5, but does not rule out the presence of epitopes in any of the other peptides. To demonstrate this it would be necessary to test a higher number of TB patients due to the genetic heterogeneity of the human population.

The expression of TB 9.5 is induced under low oxygen conditions

Immunogenic proteins may be identified by the means of their upregulation *in vivo* or in environments which reflect the *in vivo* situation. This may be different stress situations such as low oxygen. To investigate the upregulation of *M. tuberculosis* proteins during low oxygen conditions the following experiments were performed: *M. tuberculosis* H37Rv (ATCC 27240) was cultured in Sauton medium enriched with 0.5 % sodium pyruvate and 0.5 % glucose. Sterile 10 ml (Nunc, Roskilde, Denmark) polystyrene tubes or 125 ml polycarbonate Erlenmeyer flasks (Corning, Acton, MA, USA) containing 6.7 ml or 20 ml of medium, respectively, was inoculated with 2×10^6 bacteria per ml. Erlenmeyer flasks were placed in a standard 37°C shaking incubator (normal cultures), whereas tubes with tightly screwed caps (low oxygen cultures) were placed at 37°C under magnetic stirring at 100 rpm. After 3 h metabolic labelling was performed by addition of 10 μ Ci/ml of L-[35 S]-methionine and L-[35 S]-cysteine (Redivue Promix, Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). After 19 h, bacteria were harvested by centrifugation, and the medium was collected. The bacterial pellet was washed once in PBS, pH 7.4, and resuspended in 300 μ l of a suspension containing equal volumes of 0.1 mm glass beads and PBS, pH 7.4, added 0.1 % SDS and 1 mM PMSF. The bacteria were lysed for 5 min at maximum speed on a MS2 minishaker (IKA Works inc., Wilmington, NC). 20 μ l of the lysates was analysed by two-dimensional gel electrophoresis (2-D PAGE): Samples were applied to 13 cm IPG pH 4-7L strips (Amersham Pharmacia Biotech, Uppsala, Sweden) during rehydration according to the manufacturer's instructions. Focusing started at 500 V (1 h), was increased to 1000 V (1 h), and finally to 8000 V (2 h) in an IPGphor unit (Amersham Pharmacia Biotech). The second dimensional separation was performed in 10-20 % SDS-PAGE gradient gels in the Protean lixi system (Bio-Rad, Richmond, CA, USA). The gel was blotted to PVDF membrane, and the membrane was exposed to Biomax MR film (Kodak, Rochester, NY, USA) for 3-21 days. The autoradiographs were scanned and analysed by the Phoretix 2D gel analysis software (Non Linear Dynamics, Newcastle upon Tyne, United Kingdom). Spots which showed more than two-fold induction under low oxygen conditions compared to normal cultures were selected. A spot with observed

mass of approx. 12 kDa and pI of 6.3 was found to be induced under low oxygen conditions. For identification of this spot, 35 µl of the low oxygen lysate was analysed by 2-D PAGE as described above and the gel was silver stained. The relevant spot was excised and identified by MALDI-MS peptide mass fingerprinting. Four fragments corresponding to the peptides 23-29, 30-40, 75-86 and 75-88 of TB9.5 (Rv0569) were matched, giving a sequence coverage of 36 % for this protein. This result demonstrates that the TB9.5 protein is upregulated under conditions that mimics the *in vivo* situation, which indicates that this protein may be a good vaccine candidate or a therapeutic vaccine candidate.

10 Example 5: ORF13A is a serological target in TB patients

To test the potential of ORF13A as a serological antigen, sera were collected from 48 TB patients (all proven culture positive for *M. tuberculosis*) and 15 healthy BCG vaccinated controls and 19 non-BCG vaccinated healthy controls. The sera were assayed for antibodies recognizing the recombinantly produced ORF13A in an ELISA assay as follows: Each of the sera was absorbed with Promega *E. coli* extract (S37761) for 4 hours at room temperature and the supernatants collected after centrifugation. 1 µg/ml of ORF13A in Carbonatbuffer pH 9.6 were absorbed over night at 5 °C to a polystyrene plate (Maxisorp, Nunc). The plates were washed in PBS-0.05% Tween-20 and the sera applied in a dilution of 1:100. After 1 hour of incubation the plates were washed 3 times with PBS-0.05% Tween-20 and 100 µl per well of peroxidase-conjugated Rabbit Anti-Human IgA, IgG, IgM was applied in a dilution of 1:8000. After 1 hour of incubation the plates were washed 3 times with PBS-0.05% Tween-20. 100 µl of substrate (TMB PLUS, Kem-En-Tec) was added per well and the reaction stopped after 30 min with 0.2 M Sulphuric acid and the absorbance was read at 405 nm. The results are shown in figure 2A, 2B and 2C.

56% of the TB patients recognized ORF13A with an absorbance more than OD 0.3. The mean for all 48 patients was OD 0.44. In contrast only one BCG vaccinated individual recognized ORF13A slightly above the cutoff and three of the non BCG-vaccinated healthy donors recognized ORF13A, only one significant above the cutoff. The mean for BCG vaccinated individuals were OD 0.18 and for non BCG-vaccinated OD 0.3.

Table 8: Serological responses to ORF13A and the 38kDa antigen evaluated by ELISA on 48 TB patients, 15 BCG vaccinated and 19 non BCG vaccinated individuals.

Antigen	TB patients		BCG vaccinated		Non BCG vaccinated	
	Percent (n) responders	Mean of OD	Percent (n) responders	Mean of OD	Percent (n) responders	Mean of OD
ORF13A	56% (27)	0.44	7% (1)	0.18	16% (3)	0.3
38 kDa	50% (24)	0.38	20% (3)	0.21	26% (5)	0.24

In table 8 the response to ORF13A is compared to an antigen which is known as one of the best serological antigens; the 38kDa phosphate binding proteins (Luashchenko, K. P., *et al* J Immunological Methods 242 (2000) 91-100). The two proteins were tested in parallel on the same donors. The 38 kDa antigens is recognized by 50% of these TB patients and 20% of the BCG vaccinated and 26% of the non BCG-vaccinated in this study population. Thus ORF13A is recognized by more TB patients and by less of the healthy controls (both BCG vaccinated and non-vaccinated) than the 38 kDa antigen. This clearly demonstrates the potential of ORF13A as a serological antigen for the diagnosis of TB, and demonstrates that ORF13A has the potential to differentiate between BCG vaccinated and *M. tuberculosis* infected individuals something, which is not possible with the current diagnostic reagent PPD. It is well known that the antibody repertoire of TB patients is very heterogeneous and it is therefore not likely that all patients will recognize the same mycobacterial antigen, as also demonstrated by these results. It is therefore most likely that a serological kit for the diagnosis of *M. tuberculosis* infection will consist of more than one component and in this respect it will be obvious to combine ORF13A with other antigens, which are recognized by TB patients. This could be the 38 kDa antigens, but also other proteins could be included.

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Claims

1. A substantially pure polypeptide, which comprises at least one amino acid sequence selected from the group consisting of:
 - 5 (a) an amino acid sequence selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1;
 - (b) an immunogenic portion of any one of the sequences in (a); and
 - (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic.
- 10 2. A substantially pure polypeptide according to claim 1, wherein the amino acid sequence analogue has at least 80% sequence identity to any of the sequences in (a) or (b).
3. A fusion polypeptide, which comprises at least one amino acid sequence selected from
 - 15 the group consisting of:
 - (a) an amino acid sequence selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1;
 - (b) an immunogenic portion of any one of the sequences in (a); and
 - (c) an amino acid sequence analogue having at least 70% sequence identity to any
 - 20 one of the sequences in (a) or (b) and at the same time being immunogenic;
- and at least one fusion partner.
4. A fusion polypeptide according to claim 3, wherein the fusion partner comprises a polypeptide fragment selected from the group consisting of:
 - 25 (a) a polypeptide fragment derived from a virulent mycobacterium;
 - (b) a polypeptide according to claim 1; and
 - (c) at least one immunogenic portion of any of such polypeptides in (a) or (b).
5. A polypeptide, which comprises at least one amino acid sequence selected from the
 - 30 group consisting of:
 - (a) an amino acid sequence selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1;
 - (b) an immunogenic portion of any one of the sequences in (a); and
 - (c) an amino acid sequence analogue having at least 70% sequence identity to any
 - 35 one of the sequences in (a) or (b) and at the same time being immunogenic;

which is lipidated so as to allow a self-adjuvating effect of the polypeptide.

6. A substantially pure polypeptide, which comprises at least one amino acid sequence selected from the group consisting of:

- 5 (a) an amino acid sequence selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1;
- (b) an immunogenic portion of any one of the sequences in (a); and
- (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;
- 10 for use as a vaccine, as a pharmaceutical or as a diagnostic reagent.

7. Use of a polypeptide according to any of the preceding claims for the preparation of a pharmaceutical composition for diagnosis of tuberculosis.

- 15 8. Use of a polypeptide according to any of claims 1-6 for the preparation of a pharmaceutical composition.

9. An immunogenic composition comprising at least one polypeptide according to any of claims 1-6.

20

10. An immunogenic composition according to claim 9, which is in the form of a vaccine.

11. An immunogenic composition according to claim 9, which is in the form of a skin test reagent.

25

12. A nucleic acid fragment in isolated form which

- (a) comprises at least one nucleic acid sequence which encodes a polypeptide as defined in any of claims 1-6, or comprises a nucleic acid sequence complementary thereto; and/or
- 30 (b) has a length of at least 10 nucleotides and hybridizes under stringent hybridization conditions with a nucleotide sequence selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1, or a nucleotide sequence complementary to any one of these sequences; or with a nucleotide sequence selected from a sequence in (a).

35

13. A nucleic acid fragment according to claim 12, which is a DNA fragment.
14. A nucleic acid fragment according to claim 12 or 13 for use as a pharmaceutical.
- 5 15. A vaccine comprising at least one nucleic acid fragment according to claim 12 or 13, optionally inserted in a vector, the vaccine effecting *in vivo* expression of antigen by an animal, including a human being, to whom the vaccine has been administered, the amount of expressed antigen being effective to confer substantially increased resistance to tuberculosis caused by virulent mycobacteria in an animal, including a human being.
- 10 16. Use of a nucleic acid fragment according to claim 12 or 13 for the preparation of a composition for the diagnosis of tuberculosis caused by virulent mycobacteria.
- 15 17. Use of a nucleic acid fragment according to claim 12 or 13 for the preparation of a pharmaceutical composition for the vaccination against tuberculosis caused by virulent mycobacteria.
18. A vaccine for immunizing an animal, including a human being, against tuberculosis caused by virulent mycobacteria comprising as the effective component a non-pathogenic
- 20 microorganism, wherein at least one copy of a DNA fragment comprising a DNA sequence encoding a polypeptide according to any of claims 1-6 has been incorporated into the microorganism in a manner allowing the microorganism to express and optionally secrete the polypeptide.
- 25 19. A replicable expression vector, which comprises at least one nucleic acid fragment according to claim 12 or 13.
20. A transformed cell harbouring at least one vector according to claim 19.
- 30 21. A method for producing a polypeptide according to any of claims 1-6, comprising:
- (a) inserting a nucleic acid fragment according to claim 12 or 13 into a vector which is able to replicate in a host cell, introducing the resulting recombinant vector into the host cell, culturing the host cell in a culture medium under conditions sufficient to effect expression of the polypeptide, and recovering the polypeptide from the
- 35 host cell or culture medium;

- (b) isolating the polypeptide from a whole mycobacterium from culture filtrate or from lysates or fractions thereof; or
 - (c) synthesizing the polypeptide.
- 5 22. A method of diagnosing tuberculosis caused by virulent mycobacteria in an animal, including a human being, comprising intradermally injecting, in the animal, at least one polypeptide according to any of claims 1-6 or an immunogenic composition according to claim 9, a positive skin response at the location of injection being indicative of the animal having tuberculosis, and a negative skin response at the location of injection being indicative of the animal not having tuberculosis.
- 10 23. A method for immunising an animal, including a human being, against tuberculosis caused by virulent mycobacteria comprising administering to the animal at least one polypeptide according to any of claims 1-6, an immunogenic composition according to claim 9, or a vaccine according to claim 18.
- 15 24. A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide according to any of claims 1-6 in an immuno assay, or a specific binding fragment of said antibody.
- 20 25. A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide according to any of claims 1-6 in an immuno assay, or a specific binding fragment of said antibody for use as a diagnostic reagent.
- 25 26. A pharmaceutical composition which comprises an immunologically responsive amount of at least one member selected from the group consisting of:
- (a) a polypeptide selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1, or an immunogenic portion thereof;
 - 30 (b) an amino acid sequence which has a sequence identity of at least 70% to any one of said polypeptides in (a) and is immunogenic;
 - (c) a fusion polypeptide comprising at least one polypeptide or amino acid sequence according to (a) or (b) and at least one fusion partner;
 - (d) a nucleic acid sequence which encodes a polypeptide or amino acid sequence
 - 35 according to (a), (b) or (c);

- (e) a nucleic acid sequence which is complementary to a sequence according to (d);
- (f) a nucleic acid sequence which has a length of at least 10 nucleotides and which hybridizes under stringent conditions with a nucleic acid sequence according to (d) or (e); and
- 5 (g) a non-pathogenic micro-organism which has incorporated therein a nucleic acid sequence according to (d), (e) or (f) in a manner to permit expression of a polypeptide encoded thereby.

27. A method for stimulating an immunogenic response in an animal which comprises
10 administering to said animal an immunologically stimulating amount of at least one member selected from the group consisting of:

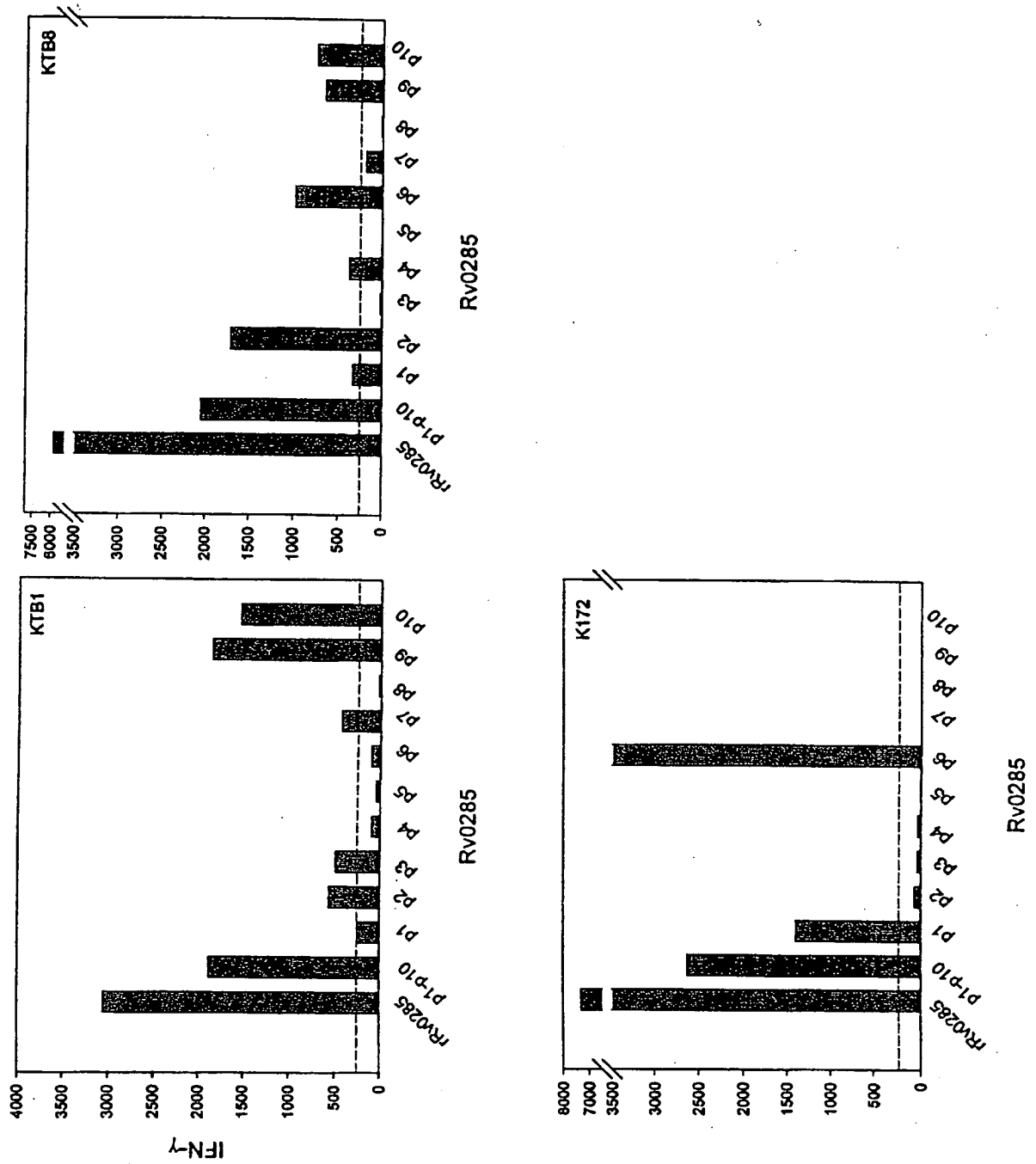
- (a) a polypeptide selected from Rv0284, Rv0285, Rv0455c, Rv0569, Rv1195, Rv1386, Rv3477, Rv3878, Rv3879c or MT3106.1, or an immunogenic portion thereof;
- 15 (b) an amino acid sequence which has a sequence identity of at least 70% to any one of said polypeptides in (a) and is immunogenic;
- (c) a fusion polypeptide comprising at least one polypeptide or amino acid sequence according to (a) or (b) and at least one fusion partner;
- (d) a nucleic acid sequence which encodes a polypeptide or amino acid sequence
20 according to (a), (b) or (c);
- (e) a nucleic acid sequence which is complementary to a sequence according to (d);
- (f) a nucleic acid sequence which has a length of at least 10 nucleotides and which hybridizes under stringent conditions with a nucleic acid sequence according to (d) or (e); and
- 25 (g) a non-pathogenic micro-organism which has incorporated therein a nucleic acid sequence according to (d), (e) or (f) in a manner to permit expression of a polypeptide encoded thereby.

28. Vaccine according to claim 15 or 18, immunogenic composition according to claim 10
30 or pharmaceutical composition according to claim 26, characterized in that said vaccine/immunogenic composition/pharmaceutical composition can be used prophylactically in a subject not infected with a virulent mycobacterium; or therapeutically in a subject already infected with a virulent mycobacterium.

29. A method for diagnosing previous or ongoing infection with a virulent mycobacterium, said method comprising:
- (a) contacting a sample with a composition comprising at least one antibody according to claim 24 or 25, at least one nucleic acid fragment according to any of claims 12-14 and/or at least one polypeptide according to any of claims 1-6; or
- (b) contacting a sample with a composition comprising at least one polypeptide according to any of claims 1-6 in order to detect a positive reaction.
30. A method of diagnosing *Mycobacterium tuberculosis* infection in a subject comprising:
- (a) contacting at least one polypeptide according to any of the claims 1-6 with a bodily fluid of the subject;
- (b) detecting binding of an antibody to said polypeptide, said binding being an indication that said subject is infected by *Mycobacterium tuberculosis* or is susceptible to *Mycobacterium tuberculosis* infection.

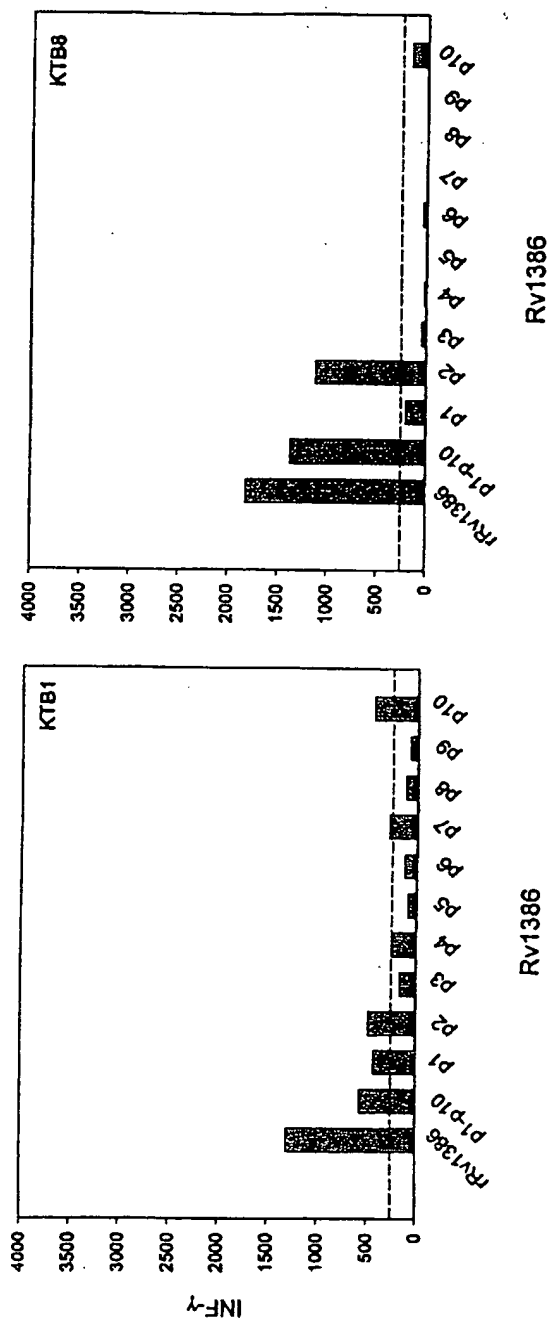
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Figure 1



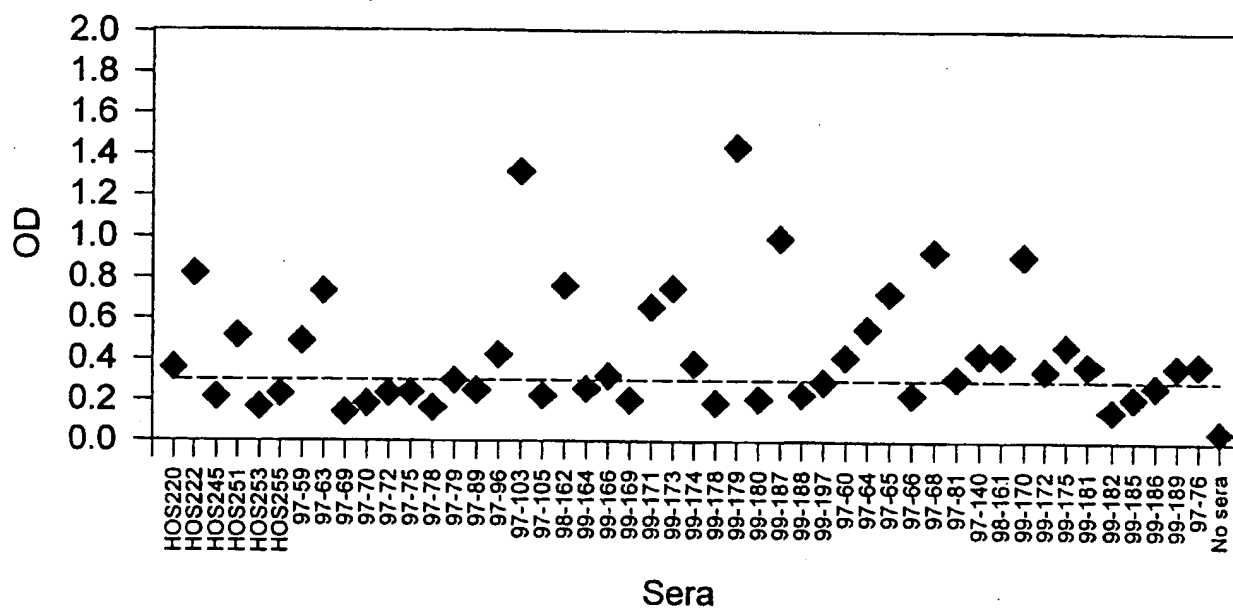
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Figure 1 - continued



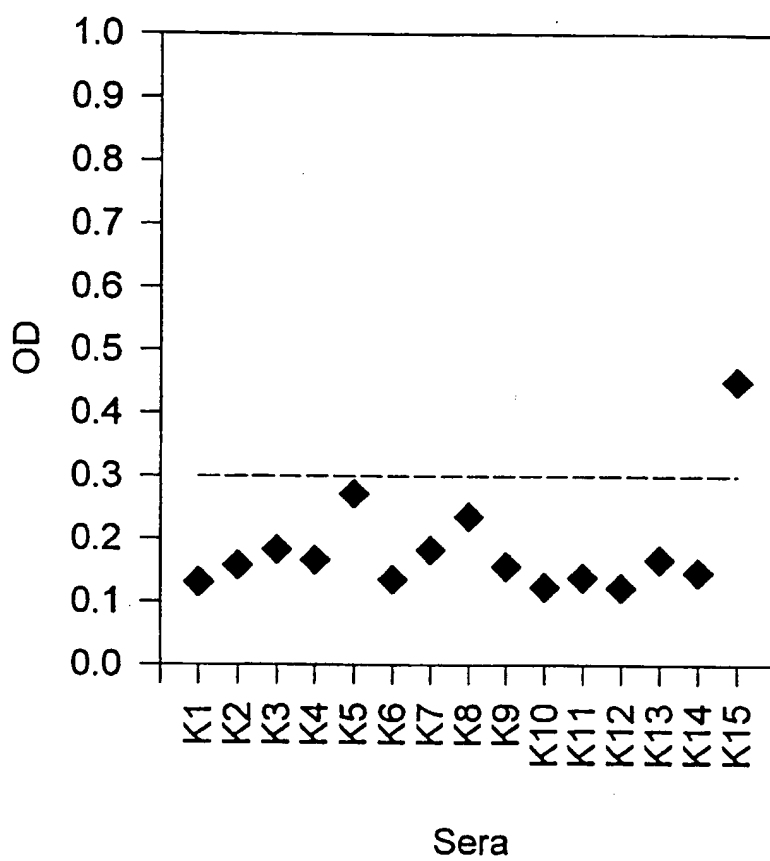
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Figure 2A



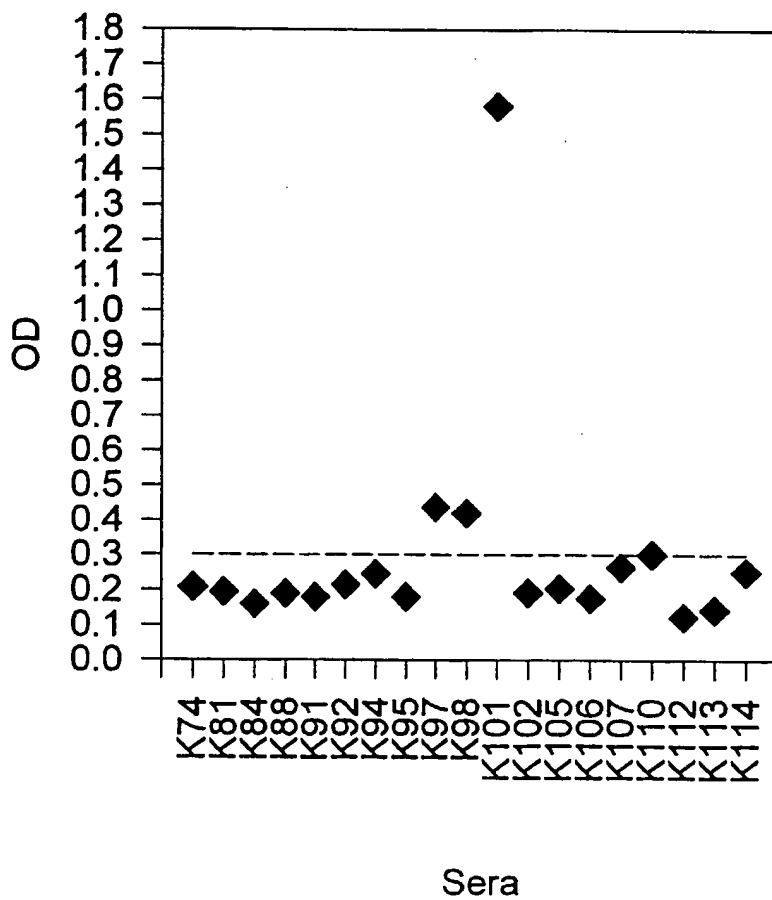
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Figure 2B



5/6

Figure 2C



6/6

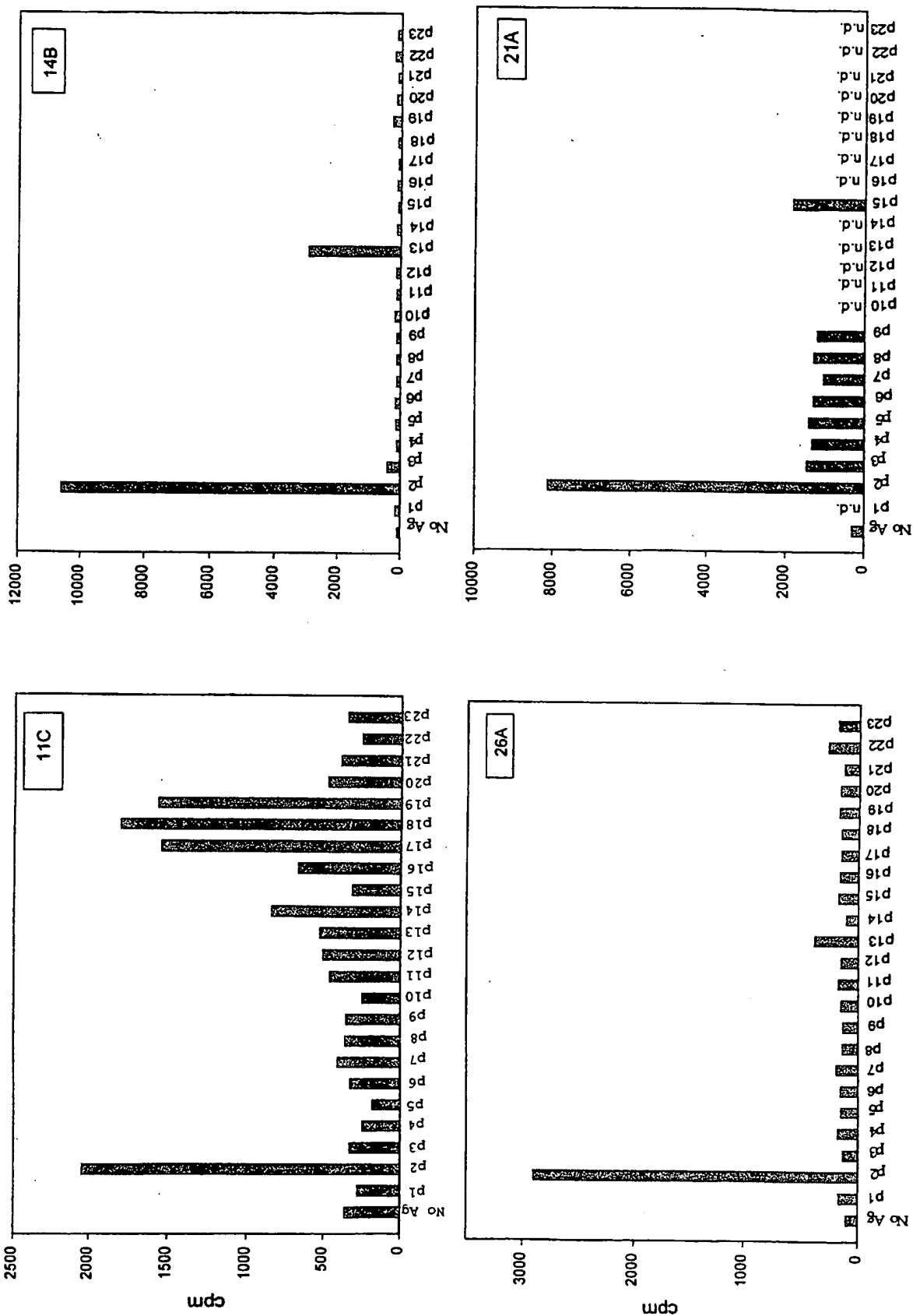


Figure 3

SEQUENCE LISTING

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<211> 3990

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20 <213> Mycobacterium tuberculosis

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<221> CDS

<222> (1)...(3990)

25 <400> 1

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1				5					10					15		
agc	cac	cag	ggc	acc	atc	atc	atc	gag	gcg	cct	ccc	gag	ctg	cct	cgg	96
Ser	His	Gln	Gly	Thr	Ile	Ile	Ile	Glu	Ala	Pro	Pro	Glu	Leu	Pro	Arg	
			20					25					30			
gtg	atc	cca	ccg	tca	ctg	cta	cga	cga	gcg	ctg	cct	tat	ctg	atc	ggg	144
Val	Ile	Pro	Pro	Ser	Leu	Leu	Arg	Arg	Ala	Leu	Pro	Tyr	Leu	Ile	Gly	
		35					40					45				
atc	ctc	atc	gtg	ggg	atg	atc	gtg	gcg	ctg	gtc	gcc	acc	ggg	atg	cgg	192
Ile	Leu	Ile	Val	Gly	Met	Ile	Val	Ala	Leu	Val	Ala	Thr	Gly	Met	Arg	
	50					55					60					
gtg	att	tct	ccg	cag	acg	ttg	ttc	ttc	cca	ttt	gtg	ctg	ctg	ttg	gcg	240
Val	Ile	Ser	Pro	Gln	Thr	Leu	Phe	Phe	Pro	Phe	Val	Leu	Leu	Leu	Ala	
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gcc	acc	gcg	ctc	tac	cgc	ggc	aac	gac	aag	aag	atg	cgc	acc	gag	gag	288
Ala	Thr	Ala	Leu	Tyr	Arg	Gly	Asn	Asp	Lys	Lys	Met	Arg	Thr	Glu	Glu	
			85					90						95		
gtc	gac	gcc	gaa	cgg	gcc	gac	tac	cta	cgt	tac	cta	tcg	gtg	gtg	cgg	336
Val	Asp	Ala	Glu	Arg	Ala	Asp	Tyr	Leu	Arg	Tyr	Leu	Ser	Val	Val	Arg	
			100					105					110			
gac	aac	att	cgg	gcc	cag	gcc	gcc	gag	cag	cgg	gcc	agc	gcg	ttg	tgg	384
Asp	Asn	Ile	Arg	Ala	Gln	Ala	Ala	Glu	Gln	Arg	Ala	Ser	Ala	Leu	Trp	
		115				120						125				
tct	cat	cct	gac	ccg	acg	gcg	ttg	gcg	tcg	gtg	ccg	ggg	tca	cgt	cgc	432
Ser	His	Pro	Asp	Pro	Thr	Ala	Leu	Ala	Ser	Val	Pro	Gly	Ser	Arg	Arg	
		130				135						140				
caa	tgg	gag	cgt	gac	ccg	cac	gac	ccc	gac	ttt	ttg	gtg	ttg	cgg	gcc	480
Gln	Trp	Glu	Arg	Asp	Pro	His	Asp	Pro	Asp	Phe	Leu	Val	Leu	Arg	Ala	
65	145					150				155					160	

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	Gly Arg His Thr Val Pro Leu Ala Thr Thr Leu Arg Val Asn Asp Thr	
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	Ala Asp Glu Ile Asp Leu Glu Pro Val Ser His Ser Ala Leu Arg Ser	
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	Leu Leu Asp Thr Gln Arg Ser Ile Gly Asp Val Pro Thr Gly Ile Asp	
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	Leu Thr Lys Val Ser Pro Ile Thr Val Leu Gly Glu Arg Ala Gln Val	
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	Arg Ala Val Leu Arg Ala Trp Ile Ala Gln Ala Val Thr Trp His Asp	
	225 230 235 240	
25	ccg acg gtg ctc ggg gtg gcg ctg gcc gcg cgt gat ctg gag ggt cgc	768
	Pro Thr Val Leu Gly Val Ala Leu Ala Ala Arg Asp Leu Glu Gly Arg	
	245 250 255	
30	gat tgg aac tgg ctg aag tgg tta ccg cac gtg gac att ccc ggc cgc	816
	Asp Trp Asn Trp Leu Lys Trp Leu Pro His Val Asp Ile Pro Gly Arg	
	260 265 270	
35	ctc atc gcg ctg ctg ggg ccc gtc ctg gca gac cgc ccg gcg ttt acc	864
	Leu Ile Ala Leu Leu Gly Pro Val Leu Ala Asp Arg Pro Ala Phe Thr	
	275 280 285 290 295 300	
40	ggg cag cca aca gat gcg ttg cgg cac ttg ctg atc gtc gtc gat gac	912
	Gly Gln Pro Thr Asp Ala Leu Arg His Leu Leu Ile Val Val Asp Asp	
	305 310 315 320	
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	Pro Asp Tyr Asp Leu Gly Ala Ser Pro Leu Ala Val Gly Arg Ala Gly	
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	Val Thr Val Val His Cys Ser Ala Ser Ala Pro His Arg Glu Gln Tyr	
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	Ser Asp Pro Glu Lys Pro Ile Leu Arg Val Ala His Gly Ala Ile Glu	
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	Arg Trp Gln Thr Gly Gly Trp Gln Pro Tyr Ile Asp Ala Ala Asp Gln	
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	Phe Ser Ala Asp Glu Ala Ala His Leu Ala Arg Arg Leu Ser Arg Trp	
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	Asp Ser Asn Pro Thr His Ala Gly Leu Arg Ser Ala Ala Thr Arg Gly	
	405 410 415	
75	gcg agt ttc acc aca ctg ctg gcc atc gag gac gca tcc cga ctg gat	1248
		1296

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	Val	Pro	Ala	Leu	Trp	Ala	Pro	Arg	Arg	Arg	Asp	Glu	Glu	Leu	Arg	Val	
			435					440					445				
10	ccg	atc	ggg	gtc	act	ggc	acc	ggc	gag	ccg	ctg	atg	ttc	gac	ctc	aaa	1392
	Pro	Ile	Gly	Val	Thr	Gly	Thr	Gly	Glu	Pro	Leu	Met	Phe	Asp	Leu	Lys	
		450					455					460					
15	gac	gaa	gcc	gag	ggc	ggg	atg	ggc	ccg	cac	ggg	ctg	atg	atc	ggc	atg	1440
	Asp	Glu	Ala	Glu	Gly	Gly	Met	Gly	Pro	His	Gly	Leu	Met	Ile	Gly	Met	
	465					470					475					480	
	acc	ggg	tcg	ggc	aag	tcg	cag	act	ttg	atg	tcg	att	ctg	ttg	tcg	ctg	1488
	Thr	Gly	Ser	Gly	Lys	Ser	Gln	Thr	Leu	Met	Ser	Ile	Leu	Leu	Ser	Leu	
					485					490					495		
20	ttg	acc	aca	cac	tcc	gcg	gag	cgg	ctc	atc	gtc	atc	tac	gcc	gac	ttc	1536
	Leu	Thr	Thr	His	Ser	Ala	Glu	Arg	Leu	Ile	Val	Ile	Tyr	Ala	Asp	Phe	
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	Tyr	Glu	Asn	Ala	Ile	Ala	Ala	Gly	His	Ser	Leu	Pro	Pro	Ile	Pro	Thr	
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	Leu	Phe	Val	Val	Ala	Asp	Glu	Phe	Thr	Leu	Met	Leu	Ala	Asp	His	Pro	
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	Glu	Tyr	Ala	Glu	Leu	Phe	Asp	Tyr	Val	Ala	Arg	Lys	Gly	Arg	Ser	Phe	
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	Ile	Glu	Ser	Gly	Lys	Glu	His	Lys	Gly	Val	Gly	Phe	Leu	Val	Pro	Ala	
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	35 40 45	
	Ile Leu Ile Val Gly Met Ile Val Ala Leu Val Ala Thr Gly Met Arg	
	50 55 60	
	Val Ile Ser Pro Gln Thr Leu Phe Phe Pro Phe Val Leu Leu Leu Ala	
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	Ala Thr Ala Leu Tyr Arg Gly Asn Asp Lys Lys Met Arg Thr Glu Glu	
	85 90 95	
	Val Asp Ala Glu Arg Ala Asp Tyr Leu Arg Tyr Leu Ser Val Val Arg	
	100 105 110	
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	Ser His Pro Asp Pro Thr Ala Leu Ala Ser Val Pro Gly Ser Arg Arg	
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	Gln Trp Glu Arg Asp Pro His Asp Pro Asp Phe Leu Val Leu Arg Ala	
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	180 185 190	
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	195 200 205	
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Leu Thr Lys Val Ser Pro Ile Thr Val Leu Gly Glu Arg Ala Gln Val
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 225 230 235 240
 5 Pro Thr Val Leu Gly Val Ala Leu Ala Ala Arg Asp Leu Glu Gly Arg
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 Asp Trp Asn Trp Leu Lys Trp Leu Pro His Val Asp Ile Pro Gly Arg
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 Glu Tyr Ala Glu Leu Phe Asp Tyr Val Ala Arg Lys Gly Arg Ser Phe
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 Arg Ile His Ile Leu Phe Ala Ser Gln Thr Leu Asp Val Gly Lys Ile
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 Pro Gly Ala Thr Pro Ile Arg Phe Arg Ser Thr Tyr Val Asp Gly Ile
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Val Ile Ala Asp Thr Asp Glu Gln Glu Pro Ala Asp Pro Pro Arg Lys
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 Ala Leu Ala Arg Ala Gly Val Gly Pro Arg Gln Trp Arg Trp Pro Leu
 785 790 795 800
 10 Gly Glu Ile Asp Arg Pro Phe Glu Met Arg Arg Asp Pro Leu Val Phe
 805 810 815
 Asp Ala Arg Ser Ser Ala Gly Asn Met Val Ile His Gly Gly Pro Lys
 820 825 830
 Ser Gly Lys Ser Thr Ala Leu Gln Thr Phe Ile Leu Ser Ala Ala Ser
 835 840 845
 15 Leu His Ser Pro His Glu Val Ser Phe Tyr Cys Leu Asp Tyr Gly Gly
 850 855 860
 Gly Gln Leu Arg Ala Leu Gln Asp Leu Ala His Val Gly Ser Val Ala
 865 870 875 880
 20 Ser Ala Leu Glu Pro Glu Arg Ile Arg Arg Thr Phe Gly Glu Leu Glu
 885 890 895
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 915 920 925
 25 Ile Asp Asn Leu Tyr Gly Phe Gly Arg Asp Asn Thr Asp Gln Phe Asn
 930 935 940
 Thr Arg Asn Pro Leu Leu Ala Arg Val Thr Glu Leu Val Asn Val Gly
 945 950 955 960
 30 Leu Ala Tyr Gly Ile His Val Ile Ile Thr Thr Pro Ser Trp Leu Glu
 965 970 975
 Val Pro Leu Ala Met Arg Asp Gly Leu Gly Leu Arg Leu Glu Leu Arg
 980 985 990
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 995 1000 1005
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 40 Thr Asn Pro Val Ala Ala Ile Asn Ala Arg Tyr Pro Gly Met Ala Ala
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 Pro Pro Val Arg Leu Leu Pro Thr Asn Leu Ala Pro His Ala Val Gly
 1060 1065 1070
 Glu Leu Tyr Arg Gly Pro Asp Gln Leu Val Ile Gly Gln Arg Glu Glu
 1075 1080 1085
 45 Asp Leu Ala Pro Val Ile Leu Asp Leu Ala Ala Asn Pro Leu Leu Met
 1090 1095 1100
 Val Phe Gly Asp Ala Arg Ser Gly Lys Thr Thr Leu Leu Arg His Ile
 1105 1110 1115 1120
 50 Ile Arg Thr Val Arg Glu His Ser Thr Ala Asp Arg Val Ala Phe Thr
 1125 1130 1135
 Val Leu Asp Arg Arg Leu His Leu Val Asp Glu Pro Leu Phe Pro Asp
 1140 1145 1150
 Asn Glu Tyr Thr Ala Asn Ile Asp Arg Ile Ile Pro Ala Met Leu Gly
 1155 1160 1165
 55 Leu Ala Asn Leu Ile Glu Ala Arg Arg Pro Pro Ala Gly Met Ser Ala
 1170 1175 1180
 Ala Glu Leu Ser Arg Trp Thr Phe Ala Gly His Thr His Tyr Leu Ile
 1185 1190 1195 1200
 60 Ile Asp Asp Val Asp Gln Val Pro Asp Ser Pro Ala Met Thr Gly Pro
 1205 1210 1215
 Tyr Ile Gly Gln Arg Pro Trp Thr Pro Leu Ile Gly Leu Leu Ala Gln
 1220 1225 1230
 Ala Gly Asp Leu Gly Leu Arg Val Ile Val Thr Gly Arg Ala Thr Gly
 1235 1240 1245
 65 Ser Ala His Leu Leu Met Thr Ser Pro Leu Leu Arg Arg Phe Asn Asp
 1250 1255 1260

	Leu Gln Ala Thr Thr Leu Met Leu Ala Gly Asn Pro Ala Asp Ser Gly	
	1265 1270 1275 1280	
	Lys Ile Arg Gly Glu Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile	
	1285 1290 1295	
5	Leu Leu Thr Asp Ser Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro	
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	Leu Val Asp Ala Ala Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser	
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	Gln Val Pro Asp Ser Pro Ala Met Thr Gly Pro Tyr Ile Gly Gln Arg	
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25	ccg tgg acc ccg ctg atc ggt ctc ctg gcc cag gcc ggc gac ttg ggg	96
	Pro Trp Thr Pro Leu Ile Gly Leu Leu Ala Gln Ala Gly Asp Leu Gly	
	20 25 30	
30	cta cgg gtg att gtc acc ggg cgt gcc act gga tcg gcg cac ctg ctg	144
	Leu Arg Val Ile Val Thr Gly Arg Ala Thr Gly Ser Ala His Leu Leu	
	35 40 45	
35	atg aca agt ccg ttg ctg cgc cgg ttc aac gac ctg cag gcg acc acg	192
	Met Thr Ser Pro Leu Leu Arg Arg Phe Asn Asp Leu Gln Ala Thr Thr	
	50 55 60	
40	ctg atg ttg gca ggc aat ccg gcc gac agc ggc aag att cgc ggt gag	240
	Leu Met Leu Ala Gly Asn Pro Ala Asp Ser Gly Lys Ile Arg Gly Glu	
	65 70 75 80	
45	cgg ttt gcc cga ttg cct gct gga cga gca att ctg ttg acc gac agt	288
	Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile Leu Leu Thr Asp Ser	
	85 90 95	
50	gat agt cca acc tac gtg cag ttg atc aac ccg ctg gtc gat gcg gcc	336
	Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro Leu Val Asp Ala Ala	
	100 105 110	
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	Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser Gln Ser	
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	<213> Mycobacterium tuberculosis	
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	20 25 30	
65	Leu Arg Val Ile Val Thr Gly Arg Ala Thr Gly Ser Ala His Leu Leu	
	35 40 45	

Met Thr Ser Pro Leu Leu Arg Arg Phe Asn Asp Leu Gln Ala Thr Thr
 50 55 60
 Leu Met Leu Ala Gly Asn Pro Ala Asp Ser Gly Lys Ile Arg Gly Glu
 65 70 75 80
 5 Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile Leu Leu Thr Asp Ser
 85 90 95
 Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro Leu Val Asp Ala Ala
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 10 Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser Gln Ser
 115 120 125

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 <211> 306
 <212> DNA
 15 <213> Mycobacterium tuberculosis

 <220>
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 Met Thr Leu Arg Val Val Pro Glu Gly Leu Ala Ala Ala Ser Ala Ala
 1 5 10 15
 25 gtg gaa gcg ctg acg gcg cgg ttg gcc gcc gcg cat gcg agc gca gcg 96
 Val Glu Ala Leu Thr Ala Arg Leu Ala Ala His Ala Ser Ala Ala
 20 25 30
 30 ccg gtg att acc gcg gta gtg ccg ccg gcg gcg gat ccg gtg tcg ctg 144
 Pro Val Ile Thr Ala Val Val Pro Pro Ala Ala Asp Pro Val Ser Leu
 35 40 45
 35 cag acc gcg gcc ggg ttc agt gca cag ggc gtc gag cac gcg gtc gtc 192
 Gln Thr Ala Ala Gly Phe Ser Ala Gln Gly Val Glu His Ala Val Val
 50 55 60
 40 acc gcc gaa ggt gtc gaa gag ctg gga cgc gcc ggc gtt ggt gtg ggc 240
 Thr Ala Glu Gly Val Glu Glu Leu Gly Arg Ala Gly Val Gly Val Gly
 65 70 75 80
 45 gaa tcc ggc gcc agc tac ctg gcc ggt gat gcg gcc gcc gcc gct acg 288
 Glu Ser Gly Ala Ser Tyr Leu Ala Gly Asp Ala Ala Ala Ala Ala Thr
 85 90 95
 tac ggg gtc gtg ggc ggc 306
 Tyr Gly Val Val Gly Gly
 100
 50
 <210> 6
 <211> 102
 <212> PRT
 <213> Mycobacterium tuberculosis
 55
 <400> 6
 Met Thr Leu Arg Val Val Pro Glu Gly Leu Ala Ala Ala Ser Ala Ala
 1 5 10 15
 60 Val Glu Ala Leu Thr Ala Arg Leu Ala Ala Ala His Ala Ser Ala Ala
 20 25 30
 Pro Val Ile Thr Ala Val Val Pro Pro Ala Ala Asp Pro Val Ser Leu
 35 40 45
 Gln Thr Ala Ala Gly Phe Ser Ala Gln Gly Val Glu His Ala Val Val
 50 55 60
 65 Thr Ala Glu Gly Val Glu Glu Leu Gly Arg Ala Gly Val Gly Val Gly 80
 65 70 75 80

[illegible]

	Thr	Glu	Asp	Phe	Pro	Ile	Pro	Arg	Arg	Met	Ile	Ala	Thr	Thr	Cys	Asp	
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	Ala	Glu	Gln	Tyr	Leu	Ala	Ala	Val	Arg	Asp	Thr	Ser	Pro	Val	Tyr	Tyr	
		50					55					60					
5	Gln	Arg	Tyr	Met	Ile	Asp	Phe	Asn	Asn	His	Ala	Asn	Leu	Gln	Gln	Ala	
	65				70						75					80	
	Thr	Ile	Asn	Lys	Ala	His	Trp	Phe	Phe	Ser	Leu	Ser	Pro	Ala	Glu	Arg	
				85						90					95		
10	Arg	Asp	Tyr	Ser	Glu	His	Phe	Tyr	Asn	Gly	Asp	Pro	Leu	Thr	Phe	Ala	
				100					105					110			
	Trp	Val	Asn	His	Met	Lys	Ile	Phe	Phe	Asn	Asn	Lys	Gly	Val	Val	Ala	
			115					120					125				
	Lys	Gly	Thr	Glu	Val	Cys	Asn	Gly	Tyr	Pro	Ala	Gly	Asp	Met	Ser	Val	
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15	Trp	Asn	Trp	Ala													
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	1				5				10						15		
	gat	caa	ccg	gac	cac	cga	ggg	ttg	att	att	gag	gtg	cgc	tca	tcc	gat	96
	Asp	Gln	Pro	Asp	His	Arg	Gly	Leu	Ile	Ile	Glu	Val	Arg	Ser	Ser	Asp	
35				20				25					30				
	ggt	tcg	ccg	ccg	tat	gtg	gtg	cgc	tgg	ctc	gag	acc	gac	cat	gtg	gcg	144
	Gly	Ser	Pro	Pro	Tyr	Val	Val	Arg	Trp	Leu	Glu	Thr	Asp	His	Val	Ala	
			35					40					45				
40	acg	gtg	att	ccg	ggt	ccg	gat	gcg	gtc	gtg	gtc	act	gcg	gag	gag	cag	192
	Thr	Val	Ile	Pro	Gly	Pro	Asp	Ala	Val	Val	Val	Thr	Ala	Glu	Glu	Gln	
		50					55					60					
	aat	gcg	gcc	gac	gag	cgg	gcg	cag	cat	cgg	ttc	ggc	gcg	gtt	cag	tcg	240
45	Asn	Ala	Ala	Asp	Glu	Ala	Gln	His	Arg	Phe	Gly	Ala	Val	Gln	Ser		
	65				70					75					80		
	gcg	atc	ctc	cat	gcc	agg	gga	acg									264
50	Ala	Ile	Leu	His	Ala	Arg	Gly	Thr									
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	Asp	Gln	Pro	Asp	His	Arg	Gly	Leu	Ile	Ile	Glu	Val	Arg	Ser	Ser	Asp	
			20					25					30				
	Gly	Ser	Pro	Pro	Tyr	Val	Val	Arg	Trp	Leu	Glu	Thr	Asp	His	Val	Ala	
			35					40					45				
65	Thr	Val	Ile	Pro	Gly	Pro	Asp	Ala	Val	Val	Val	Thr	Ala	Glu	Glu	Gln	
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 Ala Ile Leu His Ala Arg Gly Thr
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5

<210> 11
 <211> 297
 <212> DNA
 <213> Mycobacterium tuberculosis

10

<220>
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 <222> (1)...(297)

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 Val Ser Phe Val Met Ala Tyr Pro Glu Met Leu Ala Ala Ala Ala Asp
 1 5 10 15

20

acc ctg cag agc atc ggt gct acc act gtg gct agc aat gcc gct gcg 96
 Thr Leu Gln Ser Ile Gly Ala Thr Thr Val Ala Ser Asn Ala Ala Ala
 20 25 30

25

gcg gcc ccg acg act ggg gtg gtg ccc ccc gct gcc gat gag gtg tcg 144
 Ala Ala Pro Thr Thr Gly Val Val Pro Pro Ala Ala Asp Glu Val Ser
 35 40 45

30

gcg ctg act gcg gcg cac ttc gcc gca cat gcg gcg atg tat cag tcc 192
 Ala Leu Thr Ala Ala His Phe Ala Ala His Ala Ala Met Tyr Gln Ser
 50 55 60

35

gtg agc gct cgg gct gct gcg att cat gac cag ttc gtg gcc acc ctt 240
 Val Ser Ala Arg Ala Ala Ala Ile His Asp Gln Phe Val Ala Thr Leu
 65 70 75 80

gcc agc agc gcc agc tcg tat gcg gcc act gaa gtc gcc aat gcg gcg 288
 Ala Ser Ser Ala Ser Ser Tyr Ala Ala Thr Glu Val Ala Asn Ala Ala
 85 90 95

40

gcg gcc agc 297
 Ala Ala Ser

45

<210> 12
 <211> 99
 <212> PRT
 <213> Mycobacterium tuberculosis

50

<400> 12
 Val Ser Phe Val Met Ala Tyr Pro Glu Met Leu Ala Ala Ala Ala Asp
 1 5 10 15
 Thr Leu Gln Ser Ile Gly Ala Thr Thr Val Ala Ser Asn Ala Ala Ala
 20 25 30

55

Ala Ala Pro Thr Thr Gly Val Val Pro Pro Ala Ala Asp Glu Val Ser
 35 40 45
 Ala Leu Thr Ala Ala His Phe Ala Ala His Ala Ala Met Tyr Gln Ser
 50 55 60

60

Val Ser Ala Arg Ala Ala Ala Ile His Asp Gln Phe Val Ala Thr Leu
 65 70 75 80
 Ala Ser Ser Ala Ser Ser Tyr Ala Ala Thr Glu Val Ala Asn Ala Ala
 85 90 95

Ala Ala Ser

65

<210> 13

<211> 306
 <212> DNA
 <213> Mycobacterium tuberculosis

5

<220>
 <221> CDS
 <222> (1)...(306)

10

<400> 13
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 Val Thr Leu Arg Val Val Pro Glu Ser Leu Ala Gly Ala Ser Ala Ala
 1 5 10 15

15

atc gaa gca gtg acc gct cgc ctg gcc gcc gcg cac gcc gcg gcg gcc 96
 Ile Glu Ala Val Thr Ala Arg Leu Ala Ala Ala His Ala Ala Ala Ala
 20 25 30

20

ccg ttt atc gcg gcg gtc atc ccg cct ggg tcc gac tcg gtt tcg gtg 144
 Pro Phe Ile Ala Ala Val Ile Pro Pro Gly Ser Asp Ser Val Ser Val
 35 40 45

25

tgc aac gcc gtt gag ttc agc gtt cac ggt agt cag cat gtg gca atg 192
 Cys Asn Ala Val Glu Phe Ser Val His Gly Ser Gln His Val Ala Met
 50 55 60

30

gcc gct cag ggg gtt gag gag ctc gcc cgc tcg ggg gtc ggg gtg gcc 240
 Ala Ala Gln Gly Val Glu Glu Leu Gly Arg Ser Gly Val Gly Val Ala
 65 70 75 80

35

gaa tcg ggt gcc agt tat gcc gct agg gat gcg ctg gcg gcg gcg tcg 288
 Glu Ser Gly Ala Ser Tyr Ala Ala Arg Asp Ala Leu Ala Ala Ala Ser
 85 90 95

40

tat ctc agc ggt ggg cta 306
 Tyr Leu Ser Gly Gly Leu
 100

40

<210> 14
 <211> 102
 <212> PRT
 <213> Mycobacterium tuberculosis

45

<400> 14
 Val Thr Leu Arg Val Val Pro Glu Ser Leu Ala Gly Ala Ser Ala Ala
 1 5 10 15
 Ile Glu Ala Val Thr Ala Arg Leu Ala Ala Ala His Ala Ala Ala Ala
 20 25 30

50

Pro Phe Ile Ala Ala Val Ile Pro Pro Gly Ser Asp Ser Val Ser Val
 35 40 45
 Cys Asn Ala Val Glu Phe Ser Val His Gly Ser Gln His Val Ala Met
 50 55 60
 Ala Ala Gln Gly Val Glu Glu Leu Gly Arg Ser Gly Val Gly Val Ala
 65 70 75 80

55

Glu Ser Gly Ala Ser Tyr Ala Ala Arg Asp Ala Leu Ala Ala Ala Ser
 85 90 95
 Tyr Leu Ser Gly Gly Leu
 100

60

<210> 15
 <211> 294
 <212> DNA
 <213> Mycobacterium tuberculosis

65

<220>
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10 ctt cgt tcc ctg ggg gca acg ctg aag gct agc aat gcc gcc gca gcc 96
 Leu Arg Ser Leu Gly Ala Thr Leu Lys Ala Ser Asn Ala Ala Ala Ala
 20 25 30

15 gtg ccg acg act ggg gtg gtg ccc ccg gct gcc gac gag gtg tcg ctg 144
 Val Pro Thr Thr Gly Val Val Pro Pro Ala Ala Asp Glu Val Ser Leu
 35 40 45

15 ctg ctt gcc aca caa ttc cgt acg cat gcg gcg acg tat cag acg gcc 192
 Leu Leu Ala Thr Gln Phe Arg Thr His Ala Ala Thr Tyr Gln Thr Ala
 50 55 60

20 agc gcc aag gcc gcg gtg atc cat gag cag ttt gtg acc acg ctg gcc 240
 Ser Ala Lys Ala Ala Val Ile His Glu Gln Phe Val Thr Thr Leu Ala
 65 70 75 80

25 acc agc gct agt tca tat gcg gac acc gag gcc gcc aac gct gtg gtc 288
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 85 90 95

30 acc ggc
 Thr Gly 294

<210> 16

<211> 98

35 <212> PRT

<213> Mycobacterium tuberculosis

<400> 16

40 Val Ser Phe Thr Ala Gln Pro Glu Met Leu Ala Ala Ala Ala Gly Glu
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 Val Pro Thr Thr Gly Val Val Pro Pro Ala Ala Asp Glu Val Ser Leu
 35 40 45

45 Leu Leu Ala Thr Gln Phe Arg Thr His Ala Ala Thr Tyr Gln Thr Ala
 50 55 60
 Ser Ala Lys Ala Ala Val Ile His Glu Gln Phe Val Thr Thr Leu Ala
 65 70 75 80
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50 Thr Gly

<210> 17

55 <211> 840

<212> DNA

<213> Mycobacterium tuberculosis

<220>

60 <221> CDS

<222> (1)...(840)

<400> 17

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	Ala Lys Leu Ala Gly Leu Val Phe Pro Gln Pro Pro Ala Pro Ile Ala	
	20 25 30	
	gtc agc gga acg gat tcg gtg gta gca gca atc aac gag acc atg cca	144
	Val Ser Gly Thr Asp Ser Val Val Ala Ala Ile Asn Glu Thr Met Pro	
10	agc atc gaa tcg ctg gtc agt gac ggg ctg ccc ggc gtg aaa gcc gcc	192
	Ser Ile Glu Ser Leu Val Ser Asp Gly Leu Pro Gly Val Lys Ala Ala	
	50 55 60	
	ctg act cga aca gca tcc aac atg aac gcg gcg gcg gac gtc tat gcg	240
	Leu Thr Arg Thr Ala Ser Asn Met Asn Ala Ala Ala Asp Val Tyr Ala	
15	65 70 75 80	
	aag acc gat cag tca ctg gga acc agt ttg agc cag tat gca ttc ggc	288
	Lys Thr Asp Gln Ser Leu Gly Thr Ser Leu Ser Gln Tyr Ala Phe Gly	
	85 90 95	
	tcg tcg ggc gaa ggc ctg gct ggc gtc gcc tcg gtc ggt ggt cag cca	336
20	Ser Ser Gly Glu Gly Leu Ala Gly Val Ala Ser Val Gly Gly Gln Pro	
	100 105 110	
	agt cag gct acc cag ctg ctg agc aca ccc gtg tca cag gtc acg acc	384
	Ser Gln Ala Thr Gln Leu Leu Ser Thr Pro Val Ser Gln Val Thr Thr	
	115 120 125	
25	cag ctc ggc gag acg gcc gct gag ctg gca ccc cgt gtt gtt gcg acg	432
	Gln Leu Gly Glu Thr Ala Ala Glu Leu Ala Pro Arg Val Val Ala Thr	
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	gtg ccg caa ctc gtt cag ctg gct ccg cac gcc gtt cag atg tcg caa	480
	Val Pro Gln Leu Val Gln Leu Ala Pro His Ala Val Gln Met Ser Gln	
30	145 150 155 160	
	aac gca tcc ccc atc gct cag acg atc agt caa acc gcc caa cag gcc	528
	Asn Ala Ser Pro Ile Ala Gln Thr Ile Ser Gln Thr Ala Gln Gln Ala	
	165 170 175	
	gcc cag agc gcg cag ggc ggc agc ggc cca atg ccc gca cag ctt gcc	576
35	Ala Gln Ser Ala Gln Gly Gly Ser Gly Pro Met Pro Ala Gln Leu Ala	
	180 185 190	
	agc gct gaa aaa ccg gcc acc gag caa gcg gag ccg gtc cac gaa gtg	624
	Ser Ala Glu Lys Pro Ala Thr Glu Gln Ala Glu Pro Val His Glu Val	
	195 200 205	
40	aca aac gac gat cag ggc gac cag ggc gac gtg cag ccg gcc gag gtc	672
	Thr Asn Asp Asp Gln Gly Asp Gln Gly Asp Val Gln Pro Ala Glu Val	
	210 215 220	
	gtt gcc gcg gca cgt gac gaa ggc gcc ggc gca tca ccg ggc cag cag	720
	Val Ala Ala Ala Arg Asp Glu Gly Ala Gly Ala Ser Pro Gly Gln Gln	
45	225 230 235 240	
	ccc ggc ggg ggc gtt ccc gcg caa gcc atg gat acc gga gcc ggt gcc	768
	Pro Gly Gly Gly Val Pro Ala Gln Ala Met Asp Thr Gly Ala Gly Ala	
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	cgc cca gcg gcg agt ccg ctg gcg gcc ccc gtc gat ccg tcg act ccg	816
50	Arg Pro Ala Ala Ser Pro Leu Ala Ala Pro Val Asp Pro Ser Thr Pro	
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	gca ccc tca aca acc aca acg ttg	840

Ala Pro Ser Thr Thr Thr Thr Leu
275 280

- 5 <210> 18
<211> 280
<212> PRT
<213> Mycobacterium tuberculosis
- 10 <400> 18
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1 5 10 15
Ala Lys Leu Ala Gly Leu Val Phe Pro Gln Pro Pro Ala Pro Ile Ala
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15 Val Ser Gly Thr Asp Ser Val Val Ala Ala Ile Asn Glu Thr Met Pro
35 40 45
Ser Ile Glu Ser Leu Val Ser Asp Gly Leu Pro Gly Val Lys Ala Ala
50 55 60
20 Leu Thr Arg Thr Ala Ser Asn Met Asn Ala Ala Asp Val Tyr Ala
65 70 75 80
Lys Thr Asp Gln Ser Leu Gly Thr Ser Leu Ser Gln Tyr Ala Phe Gly
85 90 95
Ser Ser Gly Glu Gly Leu Ala Gly Val Ala Ser Val Gly Gly Gln Pro
100 105 110
25 Ser Gln Ala Thr Gln Leu Leu Ser Thr Pro Val Ser Gln Val Thr Thr
115 120 125
Gln Leu Gly Glu Thr Ala Ala Glu Leu Ala Pro Arg Val Val Ala Thr
130 135 140
30 Val Pro Gln Leu Val Gln Leu Ala Pro His Ala Val Gln Met Ser Gln
145 150 155 160
Asn Ala Ser Pro Ile Ala Gln Thr Ile Ser Gln Thr Ala Gln Gln Ala
165 170 175
Ala Gln Ser Ala Gln Gly Gly Ser Gly Pro Met Pro Ala Gln Leu Ala
180 185 190
35 Ser Ala Glu Lys Pro Ala Thr Glu Gln Ala Glu Pro Val His Glu Val
195 200 205
Thr Asn Asp Asp Gln Gly Asp Gln Gly Asp Val Gln Pro Ala Glu Val
210 215 220
40 Val Ala Ala Ala Arg Asp Glu Gly Ala Gly Ala Ser Pro Gly Gln Gln
225 230 235 240
Pro Gly Gly Gly Val Pro Ala Gln Ala Met Asp Thr Gly Ala Gly Ala
245 250 255
Arg Pro Ala Ala Ser Pro Leu Ala Ala Pro Val Asp Pro Ser Thr Pro
260 265 270
45 Ala Pro Ser Thr Thr Thr Thr Leu
275 280
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<211> 543
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<213> Mycobacterium tuberculosis
- <220>
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55 <222> (1)... (543)
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ccg ggc ggc tgg gtg gaa gcc gat gaa gac act ttc tat gac cgg gcc 96
Pro Gly Gly Trp Val Glu Ala Asp Glu Asp Thr Phe Tyr Asp Arg Ala
20 25 30
65 cag gaa tat agc cag gtt ttg caa agg gtc acc gat gta ttg gac acc 144

	Gln	Glu	Tyr	Ser	Gln	Val	Leu	Gln	Arg	Val	Thr	Asp	Val	Leu	Asp	Thr		
	35						40					45						
5	tgc	cgc	cag	cag	aaa	ggc	cac	gtc	ttc	gaa	ggc	ggc	cta	tgg	tcc	ggc	192	
	Cys	Arg	Gln	Gln	Lys	Gly	His	Val	Phe	Glu	Gly	Gly	Leu	Trp	Ser	Gly		
	50					55					60							
10	ggc	gcc	gcc	aat	gct	gcc	aac	ggc	gcc	ctg	ggt	gca	aac	atc	aat	caa	240	
	Gly	Ala	Ala	Asn	Ala	Ala	Asn	Gly	Ala	Leu	Gly	Ala	Asn	Ile	Asn	Gln		
	65					70					75					80		
15	ttg	atg	acg	ctg	cag	gat	tat	ctc	gcc	acg	gtg	att	acc	tgg	cac	agg	288	
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	1 5 10 15			
40	gac ggt aac cgg tcg tgc ggg tgt gtg acg cct aag gaa gga gtg tgg			96
	Asp Gly Asn Arg Ser Cys Gly Cys Val Thr Pro Lys Glu Gly Val Trp			
	20 25 30			
45	gtg gtg acg ctg aga gtg gtt cct gag ggt ttg gcg gcc gcc agt gcg			144
	Val Val Thr Leu Arg Val Val Pro Glu Gly Leu Ala Ala Ala Ser Ala			
	35 40 45			
50	gcg gtg gag gcg ttg acc gca cgg ctg gcc gcc gca cac gct ggc gcg			192
	Ala Val Glu Ala Leu Thr Ala Arg Leu Ala Ala Ala His Ala Gly Ala			
	50 55 60			
55	gcg ccg gcg att acg gcg gtg gtg gcg ccg gcg gcg gat ccg gtg tcg			240
	Ala Pro Ala Ile Thr Ala Val Val Ala Pro Ala Ala Asp Pro Val Ser			
	65 70 75 80			
60	ttg cag agt gcg gtg ggg ttt agc gcc tta ggt agc gag cat gcg gcg			288
	Leu Gln Ser Ala Val Gly Phe Ser Ala Leu Gly Ser Glu His Ala Ala			
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65	atc gcg ggc gaa ggg gtc gag gag ctg ggt cgt tcc ggg gtc gct gtg			336
	Ile Ala Gly Glu Gly Val Glu Glu Leu Gly Arg Ser Gly Val Ala Val			
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65	ggt gag tct ggg atc ggt tat gcc gcc ggt gat gcg gtg gcg gcg gcg			384
	Gly Glu Ser Gly Ile Gly Tyr Ala Ala Gly Asp Ala Val Ala Ala Ala			
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 35 40 45
 Ala Val Glu Ala Leu Thr Ala Arg Leu Ala Ala Ala His Ala Gly Ala
 50 55 60
 20 Ala Pro Ala Ile Thr Ala Val Val Ala Pro Ala Ala Asp Pro Val Ser
 65 70 75 80
 Leu Gln Ser Ala Val Gly Phe Ser Ala Leu Gly Ser Glu His Ala Ala
 85 90 95
 25 Ile Ala Gly Glu Gly Val Glu Glu Leu Gly Arg Ser Gly Val Ala Val
 100 105 110
 Gly Glu Ser Gly Ile Gly Tyr Ala Ala Gly Asp Ala Val Ala Ala Ala
 115 120 125
 Thr Tyr Leu Val Ser Gly Gly Ser Leu
 130 135

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 1 5 10 15
 His Gln Gly Thr
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40
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45
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 1 5 10 15
 50 Leu Leu Arg Arg
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55
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60
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 Ala Leu Pro Tyr Leu Ile Gly Ile Leu Ile Val Gly Met Ile Val Ala
 1 5 10 15
 Leu Val Ala Thr
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65
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<213> Mycobacterium tuberculosis

<400> 57

5 Gly Met Arg Val Ile Ser Pro Gln Thr Leu Phe Phe Pro Phe Val Leu
1 5 10 15
Leu Leu Ala Ala
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<210> 58

10 <211> 20

<212> PRT

<213> Mycobacterium tuberculosis

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15 Thr Ala Leu Tyr Arg Gly Asn Asp Lys Lys Met Arg Thr Glu Glu Val
1 5 10 15
Asp Ala Glu Arg
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20 <210> 59

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

25 <400> 59

Ala Asp Tyr Leu Arg Tyr Leu Ser Val Val Arg Asp Asn Ile Arg Ala
1 5 10 15
Gln Ala Ala Glu
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30 <210> 60

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<212> PRT

<213> Mycobacterium tuberculosis

35 <400> 60

Gln Arg Ala Ser Ala Leu Trp Ser His Pro Asp Pro Thr Ala Leu Ala
1 5 10 15
Ser Val Pro Gly
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40 <210> 61

<211> 20

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45 <213> Mycobacterium tuberculosis

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Ser Arg Arg Gln Trp Glu Arg Asp Pro His Asp Pro Asp Phe Leu Val
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Leu Arg Ala Gly
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<210> 62

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 62

Arg His Thr Val Pro Leu Ala Thr Thr Leu Arg Val Asn Asp Thr Ala
1 5 10 15
Asp Glu Ile Asp
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65 <210> 63

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

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 5 Leu Glu Pro Val Ser His Ser Ala Leu Arg Ser Leu Leu Asp Thr Gln
 1 5 10 15
 Arg Ser Ile Gly
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10 <210> 64
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15 <400> 64
 Asp Val Pro Thr Gly Ile Asp Leu Thr Lys Val Ser Pro Ile Thr Val
 1 5 10 15
 Leu Gly Glu Arg
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20 <210> 65
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25 <400> 65
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 1 5 10 15
 Trp His Asp Pro
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30 <210> 66
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35 <400> 66
 Thr Val Leu Gly Val Ala Leu Ala Ala Arg Asp Leu Glu Gly Arg Asp
 1 5 10 15
 Trp Asn Trp Leu
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40 <210> 67
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45 <400> 67
 Lys Trp Leu Pro His Val Asp Ile Pro Gly Arg Leu Asp Ala Leu Gly
 1 5 10 15
 50 Pro Ala Arg Asn
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55 <210> 68
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 <213> Mycobacterium tuberculosis

60 <400> 68
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 Ala Asp Arg Pro
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65 <210> 69
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<213> Mycobacterium tuberculosis

<400> 69

5 Ala Phe Thr Gly Gln Pro Thr Asp Ala Leu Arg His Leu Leu Ile Val
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<210> 70

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<213> Mycobacterium tuberculosis

<400> 70

15 Asp Tyr Asp Leu Gly Ala Ser Pro Leu Ala Val Gly Arg Ala Gly Val
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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 71

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Cys Ser Ala Ser Ala Pro His Arg Glu Gln Tyr Ser Asp Pro Glu Lys
 1 5 10 15
 Pro Ile Leu Arg
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<210> 72

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 72

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Val Ala His Gly Ala Ile Glu Arg Trp Gln Thr Gly Gly Trp Gln Pro
 1 5 10 15
 Tyr Ile Asp Ala
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<210> 73

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 73

45

Ala Asp Gln Phe Ser Ala Asp Glu Ala Ala His Leu Ala Arg Arg Leu
 1 5 10 15
 Ser Arg Trp Asp
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<210> 74

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 74

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Ser Asn Pro Thr His Ala Gly Leu Arg Ser Ala Ala Thr Arg Gly Ala
 1 5 10 15
 Ser Phe Thr Thr
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<210> 75

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis
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 5 Leu Leu Gly Ile Glu Asp Ala Ser Arg Leu Asp Val Pro Ala Leu Trp
 1 5 10 15
 Ala Pro Arg Arg
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 10 <211> 20
 <212> PRT
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 15 Arg Asp Glu Glu Leu Arg Val Pro Ile Gly Val Thr Gly Thr Gly Glu
 1 5 10 15
 Pro Leu Met Phe
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 <211> 20
 <212> PRT
 <213> Mycobacterium tuberculosis
 <400> 77
 25 Asp Leu Lys Asp Glu Ala Glu Gly Gly Met Gly Pro His Gly Leu Met
 1 5 10 15
 Ile Gly Met Thr
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 <211> 20
 <212> PRT
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 35 Gly Ser Gly Lys Ser Gln Thr Leu Met Ser Ile Leu Leu Ser Leu Leu
 1 5 10 15
 Thr Thr His Ser
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 50 Ala Glu Arg Leu Ile Val Ile Tyr Ala Asp Phe Lys Gly Glu Ala Gly
 1 5 10 15
 Ala Asp Ser Phe
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 <400> 80
 60 Arg Asp Phe Pro Gln Val Val Ala Val Ile Ser Asn Met Ala Glu Lys
 1 5 10 15
 Lys Ser Leu Ala
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 65 <211> 20
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<213> Mycobacterium tuberculosis

<400> 81

5 Asp Arg Phe Ala Asp Thr Leu Arg Gly Glu Val Ala Arg Arg Glu Met
1 5 10 15
Leu Leu Arg Glu
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<210> 82

10 <211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 82

15 Ala Gly Arg Lys Val Gln Gly Ser Ala Phe Asn Ser Val Leu Glu Tyr
1 5 10 15
Glu Asn Ala Ile
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20 <210> 83

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

25 <400> 83

Ala Ala Gly His Ser Leu Pro Pro Ile Pro Thr Leu Phe Val Val Ala
1 5 10 15
Asp Glu Phe Thr
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30

<210> 84

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<212> PRT

<213> Mycobacterium tuberculosis

35

<400> 84

Leu Met Leu Ala Asp His Pro Glu Tyr Ala Glu Leu Phe Asp Tyr Val
1 5 10 15
Ala Arg Lys Gly
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<210> 85

<211> 20

<212> PRT

45

<213> Mycobacterium tuberculosis

<400> 85

Arg Ser Phe Arg Ile His Ile Leu Phe Ala Ser Gln Thr Leu Asp Val
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Gly Lys Ile Lys
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<210> 86

<211> 20

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<212> PRT

<213> Mycobacterium tuberculosis

<400> 86

Asp Ile Asp Lys Asn Thr Ala Tyr Arg Ile Gly Leu Lys Val Ala Ser
1 5 10 15
Pro Ser Val Ser
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<210> 87

<211> 20

65

<212> PRT

<213> Mycobacterium tuberculosis

<400> 87

5 Arg Gln Ile Ile Gly Val Glu Asp Ala Tyr His Ile Glu Ser Gly Lys
1 5 10 15
Glu His Lys Gly
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<210> 88

10 <211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 88

15 Val Gly Phe Leu Val Pro Ala Pro Gly Ala Thr Pro Ile Arg Phe Arg
1 5 10 15
Ser Thr Tyr Val
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<210> 89

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 89

25 Asp Gly Ile Tyr Glu Pro Pro Gln Thr Ala Lys Ala Val Val Val Gln
1 5 10 15
Ser Val Pro Glu
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<210> 90

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<212> PRT

<213> Mycobacterium tuberculosis

<400> 90

35 Pro Lys Leu Phe Thr Ala Ala Ala Val Glu Pro Asp Pro Gly Thr Val
1 5 10 15
Ile Ala Asp Thr
40 20

<210> 91

<211> 20

<212> PRT

45 <213> Mycobacterium tuberculosis

<400> 91

50 Asp Glu Gln Glu Pro Ala Asp Pro Pro Arg Lys Leu Ile Ala Thr Ile
1 5 10 15
Gly Glu Gln Leu
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<210> 92

<211> 20

55 <212> PRT

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<400> 92

60 Ala Arg Tyr Gly Pro Arg Ala Pro Gln Leu Trp Leu Pro Pro Leu Asp
1 5 10 15
Glu Thr Ile Pro
20

<210> 93

65 <211> 20

<212> PRT

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<400> 93

5 Leu Ser Ala Ala Leu Ala Arg Ala Gly Val Gly Pro Arg Gln Trp Arg
1 5 10 15
Trp Pro Leu Gly
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<210> 94

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<211> 20

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<213> Mycobacterium tuberculosis

<400> 94

15 Glu Ile Asp Arg Pro Phe Glu Met Arg Arg Asp Pro Leu Val Phe Asp
1 5 10 15
Ala Arg Ser Ser
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<210> 95

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 95

25

Ala Gly Asn Met Val Ile His Gly Gly Pro Lys Ser Gly Lys Ser Thr
1 5 10 15
Ala Leu Gln Thr
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<210> 96

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<212> PRT

<213> Mycobacterium tuberculosis

35

<400> 96

Phe Ile Leu Ser Ala Ala Ser Leu His Ser Pro His Glu Val Ser Phe
1 5 10 15
Tyr Cys Leu Asp
20

40

<210> 97

<211> 20

<212> PRT

45

<213> Mycobacterium tuberculosis

<400> 97

Tyr Gly Gly Gly Gln Leu Arg Ala Leu Gln Asp Leu Ala His Val Gly
1 5 10 15
Ser Val Ala Ser
20

50

<210> 98

<211> 20

55

<212> PRT

<213> Mycobacterium tuberculosis

<400> 98

Ala Leu Glu Pro Glu Arg Ile Arg Arg Thr Phe Gly Glu Leu Glu Gln
1 5 10 15
Leu Leu Leu Ser
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60

<210> 99

65

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 99

5 Arg Gln Gln Arg Glu Val Phe Arg Asp Arg Gly Ala Asn Gly Ser Thr
1 5 10 15
Pro Asp Asp Gly
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<210> 100

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 100

15 Phe Gly Glu Val Phe Leu Val Ile Asp Asn Leu Tyr Gly Phe Gly Arg
1 5 10 15
Asp Asn Thr Asp
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<210> 101

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 101

25

Gln Phe Asn Thr Arg Asn Pro Leu Leu Ala Arg Val Thr Glu Leu Val
1 5 10 15
Asn Val Gly Leu
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<210> 102

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 102

35

Ala Tyr Gly Ile His Val Ile Ile Thr Thr Pro Ser Trp Leu Glu Val
1 5 10 15
Pro Leu Ala Met
20

<210> 103

40

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 103

45

Arg Asp Gly Leu Gly Leu Arg Leu Glu Leu Arg Leu His Asp Ala Arg
1 5 10 15
Asp Ser Asn Val
20

<210> 104

55

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 104

60

Arg Val Val Gly Ala Leu Arg Arg Pro Ala Asp Ala Val Pro His Asp
1 5 10 15
Gln Pro Gly Arg
20

<210> 105

65

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 105

5 Gly Leu Thr Met Ala Ala Glu His Phe Leu Phe Ala Ala Pro Glu Leu
1 5 10 15
Asp Ala Gln Thr
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<210> 106

10

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 106

15 Asn Pro Val Ala Ala Ile Asn Ala Arg Tyr Pro Gly Met Ala Ala Pro
1 5 10 15
Pro Val Arg Leu
20

<210> 107

20

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 107

25

Leu Pro Thr Asn Leu Ala Pro His Ala Val Gly Glu Leu Tyr Arg Gly
1 5 10 15
Pro Asp Gln Leu
20

<210> 108

30

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 108

35

Val Ile Gly Gln Arg Glu Glu Asp Leu Ala Pro Val Ile Leu Asp Leu
1 5 10 15
Ala Ala Asn Pro
20

<210> 109

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<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 109

45

Leu Leu Met Val Phe Gly Asp Ala Arg Ser Gly Lys Thr Thr Leu Leu
1 5 10 15
Arg His Ile Ile
20

<210> 110

55

<211> 20

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<213> Mycobacterium tuberculosis

<400> 110

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Arg Thr Val Arg Glu His Ser Thr Ala Asp Arg Val Ala Phe Thr Val
1 5 10 15
Leu Asp Arg Arg
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<210> 111

65

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 111

5 Leu His Leu Val Asp Glu Pro Leu Phe Pro Asp Asn Glu Tyr Thr Ala
1 5 10 15
Asn Ile Asp Arg
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<210> 112

10 <211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 112

15 Ile Ile Pro Ala Met Leu Gly Leu Ala Asn Leu Ile Glu Ala Arg Arg
1 5 10 15
Pro Pro Ala Gly
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<210> 113

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 113

25 Met Ser Ala Ala Glu Leu Ser Arg Trp Thr Phe Ala Gly His Thr His
1 5 10 15
Tyr Leu Ile Ile
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<210> 114

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 114

35 Asp Asp Val Asp Gln Val Pro Asp Ser Pro Ala Met Thr Gly Pro Tyr
1 5 10 15
Ile Gly Gln Arg
40 20

<210> 115

<211> 20

<212> PRT

45 <213> Mycobacterium tuberculosis

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50 Pro Tyr Ile Gly Gln Arg Pro Trp Thr Pro Leu Ile Gly Leu Leu Ala
1 5 10 15
Gln Ala Gly Asp
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<210> 116

<211> 20

55 <212> PRT

<213> Mycobacterium tuberculosis

<400> 116

60 Leu Ala Gln Ala Gly Asp Leu Gly Leu Arg Val Ile Val Thr Gly Arg
1 5 10 15
Ala Thr Gly Ser
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<210> 117

65 <211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 117

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1 5 10 15
Arg Arg Phe Asn
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10 <210> 118
<211> 20
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<400> 118

15 Leu Leu Arg Arg Phe Asn Asp Leu Gln Ala Thr Thr Leu Met Leu Ala
1 5 10 15
Gly Asn Pro Ala
20

20 <210> 119
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<400> 119

25 Leu Ala Gly Asn Pro Ala Asp Ser Gly Lys Ile Arg Gly Glu Arg Phe
1 5 10 15
Ala Arg Leu Pro
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30 <210> 120
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<400> 120

35 Glu Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile Leu Leu Thr Asp
1 5 10 15
Ser Asp Ser Pro
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<212> PRT

45 <213> Mycobacterium tuberculosis

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50 Leu Thr Asp Ser Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro Leu
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Val Asp Ala Ala
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<212> PRT

55 <213> Mycobacterium tuberculosis

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60 Asn Pro Leu Val Asp Ala Ala Ala Val Ser Gly Glu Thr Gln Gln Lys
1 5 10 15
Gly Ser Gln Ser
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 1 5 10 15
 Lys Leu Ala Gly
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10 <210> 124
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 1 5 10 15
 Pro Ile Ala Val
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20 <210> 125
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25 <400> 125
 Gln Pro Pro Ala Pro Ile Ala Val Ser Gly Thr Asp Ser Val Val Ala
 1 5 10 15
 Ala Ile Asn Glu
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30 <210> 126
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35 <400> 126
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 1 5 10 15
 Val Ser Asp Gly
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40 <210> 127
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45 <400> 127
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 1 5 10 15
 50 Thr Arg Thr Ala
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55 <210> 128
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<400> 128
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 1 5 10 15
 Val Tyr Ala Lys
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65 <210> 129
 <211> 20
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<213> Mycobacterium tuberculosis

<400> 129

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1 5 10 15
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<210> 130

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 130

15 Leu Gly Thr Ser Leu Ser Gln Tyr Ala Phe Gly Ser Ser Gly Glu Gly
1 5 10 15
Leu Ala Gly Val
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20 <210> 131

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

25 <400> 131

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1 5 10 15
Gln Ala Thr Gln
20

30

<210> 132

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

35

<400> 132

Gly Gln Pro Ser Gln Ala Thr Gln Leu Leu Ser Thr Pro Val Ser Gln
1 5 10 15
Val Thr Thr Gln
20

40

<210> 133

<211> 20

<212> PRT

45

<213> Mycobacterium tuberculosis

<400> 133

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      Pro Val Ser Gln Val Thr Thr Gln Leu Gly Glu Thr Ala Ala Glu Leu
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50  Ala Pro Arg Val
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50

<210> 134

<211> 20

55

<212> PRT

<213> Mycobacterium tuberculosis

<400> 134

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Gln Leu Ala Pro
20

65

<210> 135

<211> 20

<212> PRT

43

<213> Mycobacterium tuberculosis

<400> 135

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 1 5 10 15
Ala Ser Pro Ile
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10 <210> 136
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<400> 136

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1 5 10 15
Gln Gln Ala Ala
20

20 <210> 137
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<400> 137

Ser Gln Thr Ala Gln Gln Ala Ala Gln Ser Ala Gln Gly Gly Ser Gly
1 5 10 15
Pro Met Pro Ala
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<210> 138
<211> 20
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<213> Mycobacterium tuberculosis

<400> 138

Gly Gly Ser Gly Pro Met Pro Ala Gln Leu Ala Ser Ala Glu Lys Pro
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Ala Thr Glu Gln
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45      <210> 139
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      <212> PRT
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55      <210> 140
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      <212> PRT
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1 5 10 15
Ala Glu Val Val
20

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65      <210> 141
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<213> Mycobacterium tuberculosis

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1 5 10 15
Gly Ala Ser Pro
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<210> 142

10 <211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 142

15 Asp Glu Gly Ala Gly Ala Ser Pro Gly Gln Gln Pro Gly Gly Gly Val
1 5 10 15
Pro Ala Gln Ala
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<210> 143

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 143

25 Gly Gly Gly Val Pro Ala Gln Ala Met Asp Thr Gly Ala Gly Ala Arg
1 5 10 15
Pro Ala Ala Ser
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<210> 144

<211> 20

<212> PRT

<213> Mycobacterium tuberculosis

<400> 144

35 Ala Gly Ala Arg Pro Ala Ala Ser Pro Leu Ala Ala Pro Val Asp Pro
1 5 10 15
Ser Thr Pro Ala
20

<210> 145

<211> 20

<212> PRT

45 <213> Mycobacterium tuberculosis

<400> 145

50 Ser Pro Leu Ala Ala Pro Val Asp Pro Ser Thr Pro Ala Pro Ser Thr
1 5 10 15
Thr Thr Thr Leu
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<210> 146

<211> 18

55 <212> PRT

<213> Mycobacterium tuberculosis

<400> 146

60 Met Ser Arg Gln Ala Ser Arg Gln Val Ser Ile Ile Arg Ser Ala Gly
1 5 10 15
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<210> 147

65 <211> 18

<212> PRT

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 1 5 10 15
 Lys Glu
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 10 <211> 18
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 1 5 10 15
 Val Pro
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